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13. ABSTRACT ***(Maximum 200 Words)***

This report summarizes research conducted during the grant period 9/23/96-9/22/00. The overarching aim of the project was to identify factors associated with sex/gender that could impact military assignments and hearing conservation programs. These factors included sex differences in basic auditory sensitivity and susceptibility to noise-induced hearing loss (NIHL), sex differences in the ability to benefit from "sound conditioning" and pharmacologic treatment, and the effects of two endogenous steroid hormones, estradiol and progesterone, on susceptibility to NIHL. During the course of the project, we established the validity of the chinchilla as an animal model for investigating sex differences in hearing, and discovered several sex differences in auditory sensitivity and susceptibility to NIHL that are independent of noise exposure history and other confounding factors. Most notably, female chinchillas consistently developed less low-frequency hearing loss than males, even after sound conditioning. In addition, we established a clear relationship between levels of endogenous steroid hormones and susceptibility to NIHL. Females had higher and more variable levels of serum estradiol than males, and animals pre-treated with estradiol developed significantly less NIHL than controls. The results of our experiments have important implications for women and men in all branches of the military who are exposed to potentially harmful levels of noise.

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I. Introduction

This report is a summary of all research conducted between September 23, 1996 to September 22, 2000. The goal of the report is to provide a brief description of our research accomplishments with respect to the original Statement of Work. Details of specific experiments can be found in publications in Appendix I. The overarching aim of the research project was to identify factors associated with sex/gender that could impact military assignments and hearing conservation programs. These factors included sex differences in basic auditory sensitivity, sex differences in susceptibility to noise-induced hearing loss (NIHL) caused by military-type noises, sex differences in the ability to benefit from prophylactic sound conditioning exposures, and the effects of two steroid hormones, estradiol and progesterone, on susceptibility to NIHL. During the course of the project, we established the validity of the chinchilla as an animal model for investigating sex differences in hearing, and discovered several sex differences in auditory sensitivity and susceptibility to NIHL that are independent of noise-exposure history and other confounding factors. In addition, we established a clear relationship between levels of endogenous steroid hormones and susceptibility to NIHL. The results of our experiments have important implications for women and men in all branches of the military.

II. Body

Summary of task objectives for Technical Objective #1: Test auditory sensitivity of female and male chinchillas before and after noise exposure. All tasks of Technical Objective #1 in the approved Statement of Work were completed during the first two years of the project. Experiments established that (a) chinchillas show small but consistent sex differences in basic auditory sensitivity that parallel those observed in humans, (b) there is a fundamental sex difference in the response of the chinchilla cochlea to high-level noise (simulated M16 rifle fire); and (c) both female and male chinchillas benefit from prophylactic "sound conditioning" exposures. Based on our results with chinchillas, it is reasonable to believe that some gender differences in humans are related to inherent anatomical and physiological differences, rather than to differences in noise exposure history. The military should take gender differences into account when designing and implementing hearing conservation programs.

Summary of task objectives for Technical Objective #2: Test auditory sensitivity before and after noise exposure in animals treated with steroid hormones, and examine the effects of steroid hormones on noise-induced hair cell loss. In the third and fourth (unfunded) years of the project, experiments were conducted to explore the effects of steroid hormones on noise-induced threshold shifts and hair cell losses in chinchillas. Estradiol was found to be protective under the noise exposure conditions we used, whereas progesterone appeared to enhance NIHL. The findings suggest a strong link between levels of endogenous steroid hormones and an individual's susceptibility to NIHL. Additional experiments explored the effects of two pharmacological compounds, R-phenylisopropyladenosine (R-PIA) and buthionine sulfoximine (BSO) on NIHL. Collectively, the results suggest that estradiol, like R-PIA and BSO, may influence NIHL through pathways involving reactive oxygen species (ROS). These findings have important implications for preventing NIHL in military personnel.

1. METHODS

All procedures were reviewed and approved by the University of Buffalo Animal Care and Use Committee (IACUC Protocol HER0113N), and conformed to NIH guidelines for the humane treatment of laboratory animals.

Subjects and surgery

Subjects were adult long-tailed chinchillas (*Chinchilla langier*) obtained from commercial breeders. Animals were deeply anesthetized with ketamine hydrochloride (Ketaset; 56 mg/kg) and acepromazine (Promace; 0.56 mg/kg) for surgery. Tungsten electrodes were implanted into the right and/or left inferior colliculus (IC) and the rostral cranium for recording auditory evoked potentials (EVPs). Details of the surgical procedures are provided in Appendix I (McFadden et al., 1999). Following surgery, the animals recovered in a quiet animal colony for one week or more prior to testing and noise exposure.

Hearing tests

The auditory sensitivity of each animal was determined from EVP thresholds. Cubic distortion product otoacoustic emissions (CDPs) were also recorded from some animals. All testing was conducted in a sound attenuating booth (Industrial Acoustics Corp. 400) lined with sound absorbing foam panels. The awake chinchilla was placed in a custom-designed tube (Snyder and Salvi, 1994) that held its head at a constant orientation within the calibrated sound field. Stimuli for EVP testing consisted of 10 ms tones (2 ms cosine rise/fall ramp, alternating phase) at octave intervals from 0.5 to 16 kHz, presented at a rate of 19 or 21/sec. Stimulus level was incremented in 5 dB steps from below threshold to 80 dB SPL. Details of the stimuli and recording procedures can be found in Appendix I (McFadden et al., 1999).

Noise stimuli and acoustic calibration

Animals were exposed to octave band noise (OBN) with a center frequency of 0.5 kHz or 4 kHz, helicopter noise (digitized from a recording made in the passenger area of a UH-60 Blackhawk helicopter cruising at 120 knots), and/or impulse noise. The impulse noise simulated impulses created by a U.S. Army M16A1 rifle (Danielson, Henderson, Gratton, Bianchi, and Salvi, 1991). The impulses were generated digitally, attenuated (HP 350D), amplified (NAD 2200), and delivered to a compression driver (JBL 2446) coupled to a sound delivery tube (5 cm dia X 20 cm) whose end was cut at a 45° angle to broaden the range of resonance (Danielson et al., 1991). The impulse exposure consisted of 50 pairs of impulses (100 total), with 50 ms between stimuli in a pair, and 1000 ms between the onset of each pair (Henselman et al., 1994). The total duration of the impulse exposure was 50 sec. For calibration of the impulse noise, a 1/8" microphone (Bruel and Kjaer Model 4138) was placed at the position that would be occupied by a restrained animal. The voltage corresponding to a 114 dB, 250 Hz tone produced by a pistonphone coupled to the microphone was determined, and used to calculate the desired voltage for a 150 dB peak SPL signal. The attenuation was adjusted to produce the desired voltage.

Octave band noises were generated digitally, attenuated (HP 350D), amplified (NAD 2200), and delivered to a compression driver (JBL 2446) suspended from the ceiling of the sound booth. The UH-60 helicopter noise was processed by a graphic equalizer (Technics SH 8065), then routed to two band-pass filters (Krohn-Hite 3550), one set for 10-2000 Hz, the other for 1000-

10,000 Hz. Output from each band-pass filter was amplified by separate amplifiers (NAD 2200) and attenuated by separate attenuators (HP 350D). The low-frequency portion of the UH-60 helicopter noise was delivered by a woofer (Power Logic HT 615), and the high-frequency portion was delivered by the compression driver (JBL 2446). During exposure to octave band and helicopter noises, animals were placed in individual cages beneath the loudspeaker and provided free access to food and water.

Experimental protocol

After electrode implantation surgery, animals were randomly assigned to treatment or control groups. In hormone experiments, animals in treatment groups received subcutaneous injections of 17-β-estradiol or progesterone (Sigma Chemicals) dissolved in olive oil vehicle; animals in vehicle control groups received an equal volume of vehicle alone on the same schedule as hormone-treated animals. IC-EVP thresholds were measured before treatment, and at various times relative to treatment and noise exposure.

After all physiological measurements had been completed, chinchillas were deeply anesthetized with sodium pentobarbital (Somlethal, 100 mg/kg i.p.) or carbon dioxide and decapitated. The cochleas were quickly removed, stained to reveal succinate dehydrogenase activity, and post-fixed with 10% formalin (see details in Appendix I, McFadden et al., 1999). Cochleas were dissected from the apex to the base, mounted in sections in glycerin on microscope slides, coverslipped, and examined under a light microscope (400X magnification). The numbers of missing outer hair cells (OHCs) and inner hair cells (IHCs) were determined for successive segments of the organ of Corti. Individual cochleograms were constructed to show the percentage of hair cells missing as a function of distance from the apex of the cochlea. Percent hair cells missing was referenced to our lab standards based on average hair cell counts from cochleas of young, healthy chinchillas. Percent distance from the apex was converted to frequency using the frequency-place map of Greenwood (1990).

Data analyses

Data analyses were geared toward answering specific experimental questions. Typically, analyses of variance (ANOVAs) and Student t-tests were used to assess differences between means of experimental and control groups. Paired t-tests were typically used to assess differences within groups as a function of time. The dependent variables were IC-EVP thresholds and threshold shifts at various times after treatment or noise exposure. All statistical tests were evaluated using a 0.05 criterion of significance.

Estradiol assays

Blood samples were collected from deeply anesthetized chinchillas prior to cochlear histology. The blood samples were centrifuged to separate serum for estradiol assays. Samples were treated with a steroid displacement reagent to free estradiol bound to transport proteins in the serum. Estradiol levels were measured using an Enzyme Immunoassay kit (Assay Designs Inc., product number 90108). Microplates from the assay were read using a microplate reader from Bio-Rad Laboratories, Inc. (Model 3550-UV). All samples were run in triplicate to improve reliability.

2. *RESULTS AND DISCUSSION*

2.1. Basic hearing sensitivity—there are fundamental differences between females and males

Mean thresholds of a large group of female and male chinchillas are shown in Figure 1. The pattern of sex differences observed in the chinchilla closely parallels the pattern described for humans. Male chinchillas have slightly lower thresholds than females at frequencies below 2 kHz, while female chinchillas have slightly lower thresholds than males at frequencies above 2 kHz. The differences are generally small, as they are for humans, but consistent. It is interesting to note that the largest divergence in mean thresholds occurs at the highest frequency tested (16 kHz); this difference was statistically significant. It is important to keep in mind that male and female chinchillas are raised under the same conditions, so that sex differences are not confounded by prior noise exposure history or by other factors that complicate the interpretation of data from humans.

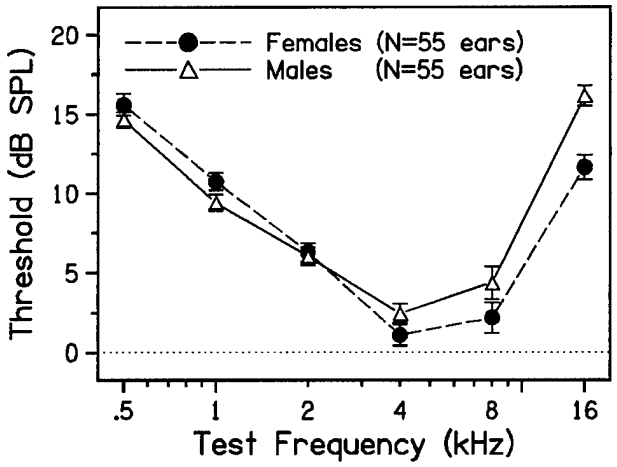

Fig.1. Mean thresholds of female and male chinchillas prior to noise exposure. Bars show standard errors of the means (SEMs).

Despite slight differences in IC-EVP thresholds (Fig. 1), there were no significant sex differences in CDPs, which reflect the status of the outer hair cell system. CDP thresholds were around 20-30 dB SPL at all frequencies, and amplitudes increased monotonically over the entire range of input levels. The lack of sex differences in CDPs suggests that the sex differences in IC-EVP thresholds are not due to differences in OHC function. Rather, threshold differences may arise from differences in stria vascularis function or activity of the olivocochlear efferent system. For a full discussion of the experiment, see reprint in Appendix IA (McFadden et al., 1999).

2.2. Sex differences in susceptibility to NIHL—Simulated M16 rifle fire produces a different pattern of hearing loss and cochlear damage for male and female chinchillas

Gender differences have been reported in susceptibility to NIHL, both temporary (e.g., Axelsson and Lindgren, 1981; Dengerink et al., 1984; Petiot and Parrot, 1984; Ward, 1966) and permanent (e.g., Berger, Royster and Thomas, 1978; Gallo and Glorig, 1964). In general, experimental studies of TTS in humans have found that males exhibit more TTS than females from low-frequency exposures (below 2 kHz), whereas females exhibit more TTS than males from high-frequency exposures (above 2 kHz). In an early investigation of gender differences in susceptibility to TTS produced by high intensity tones and noise, Ward (1966) conducted 17 experiments with 24 male and 25 female adults. Females showed approximately 30% less TTS than males when the exposure frequency was below 1 kHz, but approximately 30% more TTS when the exposure frequency was above 2 kHz.

The above studies examined TTS rather than the more important issue of PTS simply because it is not ethical to intentionally induce PTS in human subjects. Most of what little we know about gender differences in PTS comes from retrospective studies of workers exposed to noise in industrial settings. Under these conditions, which typically involve exposure to low-frequency continuous noises, males typically develop much more hearing loss than females. Both Berger et al. (1978) and Gallo and Glorig (1964), for instance, found approximately 20 dB more PTS in males than in females after 9 years of industrial noise exposure. These results are consistent with the gender differences observed in Ward's (1966) studies of TTS. However, there are no comparable studies of gender differences in PTS produced by exposures to high-level impulse noises that are more typical of military exposures.

Our results from Control Group animals provide a perspective on sex differences in susceptibility to NIHL from high-level impulse noise. Prior to noise exposure, females exhibited slightly lower thresholds than males at 8 and 16 kHz. When tested 15 min after exposure to 150 dB peak SPL impulse noise, both females and males exhibited significant threshold elevations (47-68 dB) at all frequencies. Males showed approximately 5-6 dB more TS than females at 0.5 and 1 kHz, whereas females showed approximately 6 dB more TS than males at 8 and 16 kHz. There was a significant Sex X Frequency interaction. For females, the TS function was curvilinear; TS increased progressively and significantly from 0.5 kHz to 4 kHz, was equivalent at 4 and 8 kHz, then declined significantly at 16 kHz. In contrast, males showed a much flatter pattern of TS, with equivalent TS at 1, 2, 4, and 8 kHz.

Relatively little threshold recovery occurred during the first day after exposure. A two-way ANOVA yielded a significant Sex X Frequency interaction. Whereas females had equivalent TS at 2, 4, 8 and 16 kHz and less TS at 0.5 and 1 kHz, males had equivalent TS at 1, 2, 4 and 8 kHz, and significantly less TS at 0.5 and 16 kHz. Significant recovery had occurred by 5 days post-exposure. Females exhibited approximately 10 dB more TS than males at 8 and 16 kHz at this time. Thresholds showed additional improvement at 30 days post-exposure, when permanent hearing loss was assessed (Fig. 2). High-level exposure produced significant PTS at all frequencies for both females and males (paired t-tests; all p values < 0.001). PTS ranged from 23-43 dB, with females showing 2-7 dB less PTS than males at low frequencies (0.5-2 kHz), and approximately 9 dB more PTS at 8 and 16 kHz . A significant Sex X Frequency interaction was obtained. Follow-up analyses indicated that both males and females developed progressively

and significantly greater PTS from 0.5 to 2 kHz. However, PTS peaked at 2 kHz for males, and declined significantly at higher frequencies. Females, in contrast, had significantly greater PTS at 8 and 16 kHz than at lower frequencies.

CDP data were consistent with the IC-EVPs. Before noise exposure, CDP I/O functions were similar for males and females. After exposure, CDP thresholds were elevated and amplitudes were significantly depressed. There was a trend for males to have lower amplitude CDPs than females at low frequencies (where males had greater PTS), but higher CDP amplitudes at high frequencies (where males had less PTS).

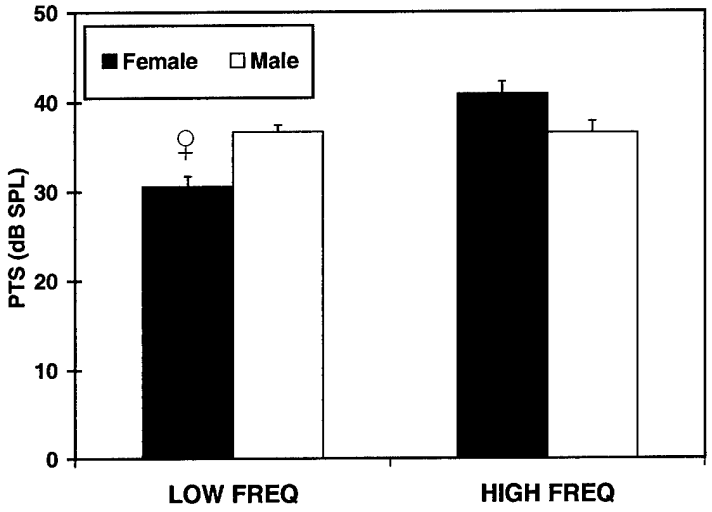

Fig. 2. PTS at low frequencies (average of 0.5, 1 and 2 kHz) and high frequencies (average of 4, 8 and 16 kHz) caused by exposure to simulated M16 rifle fire (impulse noise at 150 dB peak SPL). Symbol (♀) denotes significant difference, $p < 0.05$.

Given the results of physiological testing (IC-EVPs and CDPs), the results of cochlear histology were somewhat surprising (see McFadden et al., 1999, Appendix IA for mean cochleograms). All ears had OHC losses ranging from 70-100% in the basal half of the cochlea, but overall, females had approximately 20% less OHC loss than males (Fig. 3). IHC losses for males peaked in the 2-3 kHz region of the cochlea, with an average loss of approximately 80%. In contrast, average IHC losses for the females did not exceed 30% in any region of the cochlea.

In summary, female chinchillas developed more high-frequency PTS, but less low-frequency PTS than males from simulated M16 rifle fire. However, males had slightly more OHC loss and substantially more IHC loss than females. The results closely parallel results from human females and males in that females appear to be more susceptible to high-frequency hearing loss,

while males appear to be more susceptible to low-frequency hearing loss from the same impulse noise exposure. For details of the experiment and an expanded discussion of the results, see Appendix IA (McFadden et al., 1999).

2.3. Establishing an effective sound conditioning paradigm—0.5 kHz OBN provides protection from impulse noise, whereas UH-60 helicopter noise does not

2.3.1. Exposure to helicopter noise does not protect the ear from M16 rifle fire

Groups of chinchillas were exposed to helicopter noise at 90 dB for 5 days, 112 dB SPL for 5 days, or 112 dB SPL for 10 days (1.5 hr/day in all three experiments). The helicopter noise is a relatively broadband stimulus, weighted toward lower frequencies (see Appendix ID for the spectrum of the noise). A level of 112 dB SPL represents the sound level that would be experienced by a soldier seated in the passenger cabin of the helicopter, while the lower level of 90 dB simulates the experience of the same soldier wearing hearing protectors. As described more fully in Appendix ID (McFadden et al., 1999 ARO presentation), neither a 5-day series of exposures to 90 or 112 dB helicopter noise, nor a 10-day series of exposures to 112 dB helicopter noise provided significant protection from impulse noise. For example, animals exposed for 5 days at 112 dB showed 3 dB of protection from PTS at low frequencies, but 3 dB more PTS at high frequencies. For all helicopter noise exposure conditions, the pattern of PTS was identical to that shown in Figure 2, and the magnitude of loss was either equivalent or greater (see McFadden et al., 1999, Appendix IE).

With respect to hair cell loss, exposure to the helicopter noise clearly exacerbated damage from impulse noise. For example, ears exposed to 90 dB helicopter noise followed by M16 rifle fire sustained approximately 20% more IHC damage in the basal 10% of the cochlea than ears exposed to impulse noise alone. Compared to the 90 dB exposure, which produced approximately 20% and 30% basal IHC losses for females and males, respectively (note larger losses in ears of males), the 112 dB exposure produced basal IHC losses of approximately 40-50%. Thus, the cochleograms indicate that long-term exposure to high-level helicopter noise produces more IHC damage to the base of the cochlea than impulse noise alone. The helicopter noise may have produced basal damage independently, or it may have potentiated the effects of impulse noise. (Note that additional IHC losses that are apparent in cochleograms are not reflected audiometrically. This is because auditory thresholds are surprisingly insensitive to IHC loss; see McFadden et al., 1998). In summary, it is clear from PTS data and cochleograms that the helicopter noise has no practical value as a sound conditioning stimulus, for preventing subsequent damage from M16 rifle fire.

2.3.2. A "standard" low-frequency conditioning noise provides significant protection for both females and males

Unlike the broadband helicopter noise, a "standard" low-frequency conditioning noise (0.5 kHz OBN at 90-95 dB SPL) provided significant protection from subsequent exposure to M16 rifle fire (see Appendix ID, McFadden et al., 2000). The 5-day conditioning regimen we used produced an average of 5-12 dB protection from PTS, with greater protection at low frequencies than at high frequencies (Fig. 3). There were some sex differences in protection, but these were relatively minor: conditioned females showed 5-10 dB more protection at individual frequencies

than conditioned males, and conditioned females showed slightly less hair cell loss than conditioned males.

IC-EVP thresholds were measured after the first and last days of exposure to the conditioning noise, and again after 5 days of recovery. Conditioning noise produced significant threshold elevations at all frequencies below 16 kHz on Day 1 for both males and females (paired t-tests, all p values <0.04). Threshold shifts decreased significantly at 0.5 kHz between Day 1 and Day 5 of conditioning for both males and females. The decreases in TS that occurred between the last day of conditioning and 5 days later were significant at all frequencies (all p values < 0.04), except at 8 kHz for males.

After 5 days of recovery from conditioning, thresholds were within 5 dB of pre-exposure values at all frequencies. At this time, animals were exposed to impulse noise (150 dB peak SPL), and thresholds were measured at 15 min, 24 hr, and 5 days after exposure. The pattern of TS seen in conditioned animals was very similar to that seen in control animals, i.e., greater TS at low frequencies for males and greater TS at high frequencies for females. Two-way (Sex X Time) mixed ANOVAs for TS at each frequency revealed a significant Sex X Time interaction at 2 kHz. The interaction occurred because females showed more recovery at 2 kHz over time than males. TS at 2 kHz was equivalent for females and males at 15 min and 24 hr, but females showed approximately 10 dB less TS at 5 days post exposure, and 15 dB less PTS than males.

When PTS was assessed, males exhibited 5-15 dB more PTS than females at frequencies below 16 kHz, and 10 dB less PTS than females at 16 kHz. To evaluate sex differences in PTS, values representing low-frequency PTS (average at 0.5, 1, and 2 kHz) and high-frequency PTS (average at 4, 8 and 16 kHz) were computed (Fig. 3). Females showed significantly less low-frequency PTS than males ($p = 0.016$), whereas the difference in high-frequency PTS was not statistically significant.

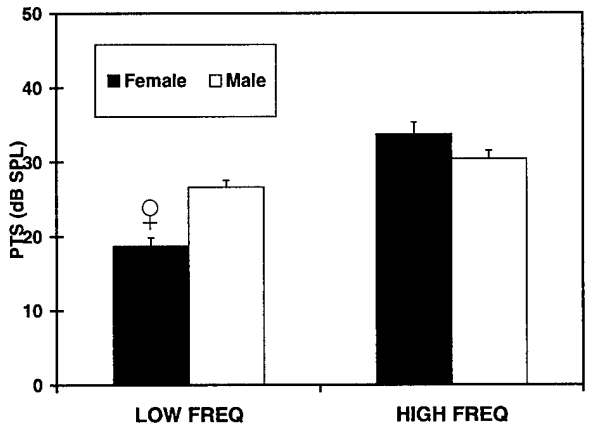

Fig. 3. PTS at low frequencies (average of 0.5, 1 and 2 kHz) and high frequencies (average of 4, 8 and 16 kHz) in animals exposed to 0.5 kHz OBN at 90-95 dB SPL for 5 days, followed by exposure to simulated M16 rifle fire. Symbol (♀) denotes significant difference, p < 0.05.

To summarize the IC-EVP test results, females consistently showed greater TS than males during sound conditioning, but thresholds of both sexes were essentially normal within 5 days after conditioning. Subsequent exposure to M16 rifle fire resulted in a pattern of TS that resembled that shown by control animals, i.e., more TS at low-frequencies for males, and more TS at high frequencies for females. When PTS was assessed 20 days after exposure to M16 rifle fire, females showed significantly less low-frequency PTS than males (Fig. 3).

The mean cochleograms showed slightly less hair cell loss in female cochleas than in male cochleas. Mean OHC losses in the apical half of the cochlea were approximately 30% for females and 40% for males. Average OHC losses in the basal half were approximately 50% for females and 60% for males. The most striking difference was seen in the 1-3 kHz region of the cochlea, where males showed approximately 30% more OHC loss than females; this was the frequency region with the largest sex differences in PTS (15 dB at 2 kHz).

2.4. Using a standard conditioning noise to protect the ear from M16 rifle fire

The experiments described above established that animals could be sound conditioned with a low-frequency (0.5 kHz) octave band noise, but not helicopter noise. A perspective on the degree of protection conferred by the low-frequency sound conditioning stimulus is provided in Figure 4. The 0.5 kHz OBN provided up to 18 dB protection for females and up to 10 dB

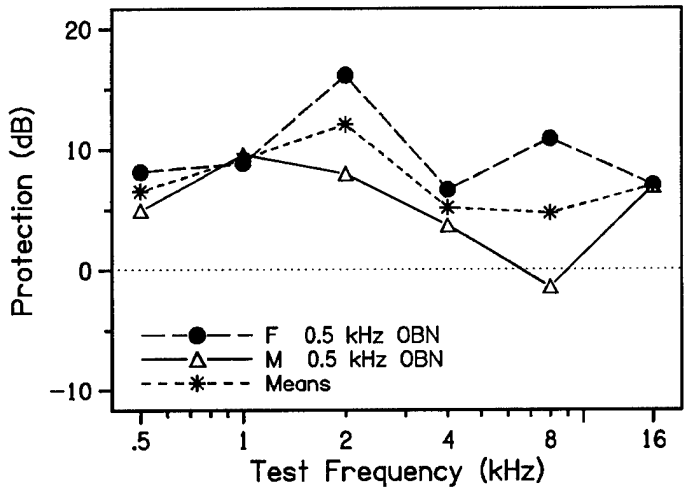

Fig. 4. Protection from NIHL afforded by a "standard" conditioning noise. Animals were sound conditioned with 0.5 kHz OBN at 90-95 dB SPL for 5 days (6 hr/day), rested for 5 days, then exposed to simulated M16 rifle fire. Differences in PTS between conditioned animals and their respective controls represent conditioning-induced protection.

protection for males at individual frequencies. Collapsed across sex, the protective effect was 5 to 12 dB across frequencies, with greater protection at low frequencies than at high frequencies. The results are described in detail in Appendix ID (McFadden et al., 2000).

Cochleograms from conditioned animals and controls provided further evidence that the low-frequency conditioning was protective (see McFadden et al., 2000, Appendix ID). OHC losses were approximately 30% less after conditioning for both females and males, and males also showed a substantial reduction of IHC loss.

The results from sound conditioning experiments clearly indicate a protective effect of the 5-day 0.5 kHz OBN conditioning regimen. In a previous study in our lab, Henselman et al. (1994) found that a 10-day series of conditioning exposures protected ears from impulse noise damage. Differences in PTS between conditioned animals and controls in Henselman's study were approximately 7-23 dB across frequencies, with the greatest protection at 2 and 4 kHz. Henselman's data were not analyzed on a frequency by frequency basis. However, an examination of their PTS data suggests that the 10-day exposure paradigm resulted in approximately 15 dB protection from low-frequency PTS, and 20 dB protection from high-frequency PTS. Thus, while both conditioning protocols produce significant protection from subsequent exposure to M16 rifle fire, the 10-day regimen provides approximately 5-10 dB more protection than the 5-day regimen used in the current study.

In summary, animals exposed to 0.5 kHz OBN for 6 hr/day for 5 days showed less PTS and hair cell loss following exposure to M16 rifle fire than control animals, particularly at low frequencies. With regard to sex differences, conditioned females showed significantly greater resistance to low-frequency PTS and less OHC loss than conditioned males. However, the magnitude of the sex difference in protection was relatively small and unlikely to have practical consequences.

2.5. Pharmacological experiments implicate reactive oxygen species in the etiology of NIHL

Recent studies from our lab and others have implicated reactive oxygen species (ROS) and free radicals as causative agents in NIHL. One line of evidence comes from studies showing that levels of ROS in the cochlea are elevated after noise exposures (Jacono et al., 1998; Yamane et al., 1995a,b; Nicotera et al., 1999, 2000). A second line of evidence is provided by studies showing that pharmacological (Hu et al., 1997; Seidman et al., 1993; Yamasoba et al., 1998, 1999) and genetic (McFadden et al., 2000a,b; Ohlemiller et al., 1999, 2000) manipulation of antioxidants in the cochlea can influence NIHL magnitude. High levels of noise are likely to create ROS by at least two pathways. First, high levels of noise tax the mitochondrial respiratory process, leading to increased free radical generation (Hyde and Rubel, 1995). Secondly, noise exposures lead to cochlear ischemia, with the consequence of increased free radical generation in the region of the stria vascularis (Yamane et al., 1995a,b).

2.5.1. NIHL from continuous noise exposures can be modulated with pharmacological agents

In one experiment, we used R-N6-phenylisopropyladenoisine (R-PIA), an adenosine receptor agonist, as a prophylactic treatment before exposure to high-level continuous noise (Hu et al., 1997; Henderson et al., 1999, Appendix IB). Briefly, a drop of saline was placed on one round window and R-PIA was applied to the other round window. One hour later, the chinchillas were exposed to a 4 kHz octave band noise at 105 dB for 4 hours. The R-PIA-treated ears recovered faster and more completely and had smaller hair cell lesions than control ears. In a complimentary experiment (Henderson et al., 1999, Appendix IB), the same general experimental procedures were used, except the experimental treatment was buthionine

sulfoximine (BSO), a drug that inhibits the synthesis of glutathione, one of the most important antioxidants in the cochlea. There were no differences between the ears treated with the small dose of BSO and the saline-treated control ears immediately after the exposure. However, at 4 days post-exposure, differences were apparent in three different measures obtained from separate groups of animals: threshold shifts, glutathione levels in the hair cells (assessed by mercury orange staining of intracellular thiols), and biochemical measures of the specific activity levels of an enzyme necessary for glutathione synthesis. BSO-treated ears had greater TS, reduced glutathione staining in outer hair cells, and lower levels of synthetic enzyme activity than control ears. Despite the clear differences between BSO- and saline-treated ears at 4 days post-exposure, there were no significant differences between ears in either PTS or hair cell loss at 20 days post-exposure. The lack of permanent effects of BSO treatment in this experiment was most likely due to the small dose of BSO used, and the short amount of time it was allowed to penetrate into the cochlea. Nevertheless, the results show that treatment with a drug that inhibits glutathione synthesis in the cochlea alters the pattern of recovery from a traumatic noise exposure. The significant protection afforded by R-PIA and the altered pattern of recovery with BSO are consistent with the hypothesis that NIHL is partially related to the action of toxic free radicals.

The results described above raise the possibility that NIHL can be reduced or prevented by increasing the levels of endogenous antioxidants of the cochlea through certain prophylactic sound conditioning exposures, or by direct drug intervention. In the following experiment, we looked at the effectiveness of R-PIA in reducing NIHL from impulse noise.

2.5.2. R-PIA protects the ear from impulse noise

The effectiveness of R-PIA in protecting the ear from the damaging effects of continuous noise prompted us to examine the issue of whether R-PIA could also attenuate hearing loss and hair cell loss caused by impulse noise. The results of the experiment described here are very exciting, as they suggest that pre-treatment with R-PIA renders the ear more resistant to the mechanical damage associated with impulse noise. Moreover, they provide additional evidence that the male cochlea is more susceptible to NIHL than the female cochlea.

R-PIA was applied to the round window membrane of the right ear, and saline or nothing was applied to the round window membrane of the left ear. Pre-exposure thresholds were measured, then animals were exposed to impulse noise. Data from the left ears show the response of the ear to the impulse noise alone, whereas data from the right ears of the same animals show the effects of R-PIA in reducing damage.

Pre-exposure thresholds were similar for males and females. Females had slightly higher thresholds than males at 0.5-4 kHz by 5-10 dB, and equivalent thresholds at 8 and 16 kHz. PTS was measured 20 days after impulse noise exposure (Fig. 5). Due to the small sample size (2 females, 5 males), statistical tests were not performed. However, three aspects of the results are important to note. First, females consistently showed less PTS than males. For both R-PIA-treated ears and control ears, sex differences were on the order of 15-35 dB. Second, pre-treatment with R-PIA clearly reduced PTS. R-PIA pre-treatment resulted in a 10-15 dB reduction of PTS across frequencies. Third, R-PIA pre-treatment was equally effective for females and males. That is, the magnitude of protection was similar for both sexes.

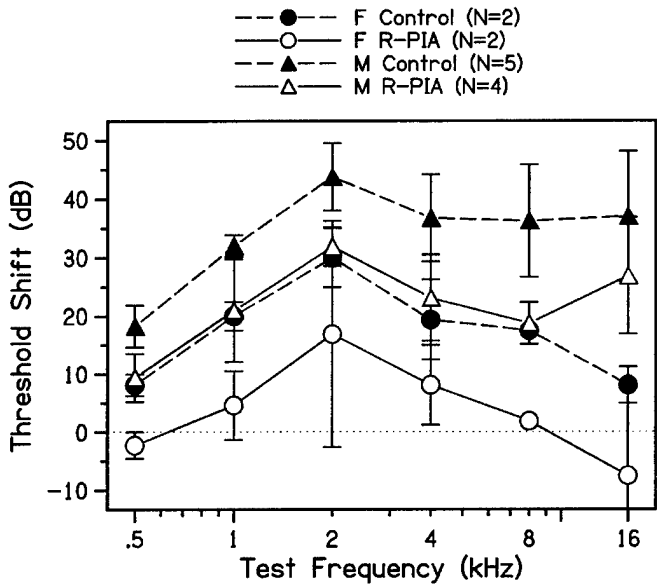

Fig. 5. PTS measured 20 days after impulse noise exposure (146 dB pSPL). Experimental animals received R-PIA prior to exposure.

The protection afforded by the R-PIA treatment may be due to several factors, as R-PIA has multiple effects: it is an adenosine agonist, an inhibitor of glutamate production, and a promoter of blood flow through stimulation of nitric oxide (NO) production. Each of these effects can influence the degree of hearing loss caused by exposure to noise. For example, the activation of the adenosine receptor can lead to an increase in glutathione and a resultant decrease in toxic ROS (Ford et al., 1997; Bobbin et al., 1995). By inhibiting glutamate production, R-PIA can reduce glutamate excitotoxicity at the synapse between the inner hair cells and the afferent nerve fibers that innervate them. Finally, R-PIA may partially counteract the cochlear ischemia that is produced by acoustic overexposure by promoting local blood flow through stimulation of NO production. Future studies should target the specific mechanism of R-PIA in order to maximize the protective effects (see Appendix IB, Henderson et al., 1999 for further discussion).

2.6. Estradiol assays: female chinchillas have higher and more variables levels of serum estradiol than male chinchillas

On average, female chinchillas had 240.12 (± 76.9) picograms of estradiol/ml serum, compared to 65.03 (± 17.5) picograms of estradiol/ml serum for males (see Appendix IF, Lockwood et al., 2000). Two aspects of these results are significant. First, the serum estradiol levels for female and male chinchillas are in the range reported for humans. This validates the use of the chinchilla in studies of hormone effects on hearing. Second, females have higher levels and are much more variable than males. This supports the idea that sex differences in chinchillas (and humans) could be due to levels of endogenous steroid hormones. Because individuals show a wide range of variability, it will be possible in future studies to correlate

endogenous levels of hormones with susceptibility to NIHL in both treated and untreated groups of animals.

2.7. Experiments with estradiol pre-treatment—estradiol is protective against NIHL

The effects of 17-β-estradiol on susceptibility to NIHL were studied in two experiments (see Lockwood et al., 2000, Appendix IF for details). In Experiment I, chinchillas in the estradiol group (N=10) were given daily injections of estradiol in olive oil for 1-2 weeks before exposure, for total doses of 200-265 mg. Thresholds measured on Days 2, 4 and 7 during the course of hormone treatment were not different from those measured before treatment, indicating that short-term estradiol treatment has no direct effect on auditory sensitivity. Animals in the control group (N=10) received either injections of the olive oil vehicle alone (n=4) or no injections (n=6). There were no significant differences between groups prior to noise exposure. All animals were exposed to impulse noise, and IC-EVPs were measured 15 min, 24 hr, 7 days and 14 days later.

Mean PTS values of and estradiol-treated animals and controls are shown in Figure 6. The animals in the vehicle control group had PTS ranging from approximately 20 dB at 0.5 kHz to 40 dB at 2 kHz. Chinchillas pre-treated with estradiol had significantly less PTS at high frequencies, by approximately 15-20 dB. These results indicate that estradiol treatment provides significant protection from threshold shifts caused by impulse noise.

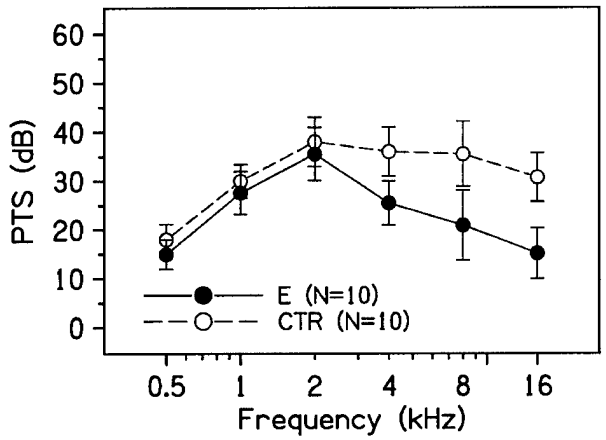

Fig. 6. PTS of estradiol-treated animals (filled symbols) and controls (open symbols) after exposure to impulse noise. Bars show SEMs.

In Experiment II, chinchillas in the estradiol groups received estradiol for 3 days or 7 days prior to exposure. Total estradiol dose was either 100 μg or 725 μg. Animals in a control group received no treatment prior to noise. There were no significant differences among groups at any frequency prior to noise exposure. All animals were exposed to 4 kHz octave band noise at a level of 105 dB SPL for 4 hours. This is the same exposure that was used in our R-PIA/continuous noise experiment (Hu et al., 1997; Henderson et al., 1999, Appendix IB).

Consequently, the effects of estradiol treatment are compared to those from R-PIA to provide a perspective on the magnitude of protection afforded by systemic treatment with estradiol.

Recovery curves were very similar for animals in the low dose and the high dose groups. However, animals in the 7-day high dose group had less threshold shift at high frequencies than animals in the low dose group or the 3-day high dose group. Differences between the 7-day high-dose E group and the other two groups were significant at 8 and 16 kHz on Day 7.

Figure 7 shows PTS as a function of frequency for treated animals and controls. Results from Hu et al. (1997) are shown for comparison. All doses of estradiol produced some degree of protection at high frequencies (note that the 4 kHz OB noise used in the experiment does not produce significant PTS at 0.5 or 1 kHz). The effects were ordered such that the greatest protection was achieved with a 7-day high dose (725 μg) of E, followed by a 7-day low dose (100 μg) of E. The 7-day high dose of estradiol provided slightly better protection than the R-PIA treatment used in the Hu et al. (1997) experiment. This is important, because the protective effects achieved with R-PIA involved invasive surgery to apply the drug to the round window membrane of the inner ear, whereas the protective effects of estradiol were achieved with simple systemic treatment. The relative ineffectiveness of the 3-day high-dose treatment suggests that estradiol is acting through slow-acting genomic mechanisms, involving protein synthesis. This process is illustrated schematically in Figure 8.

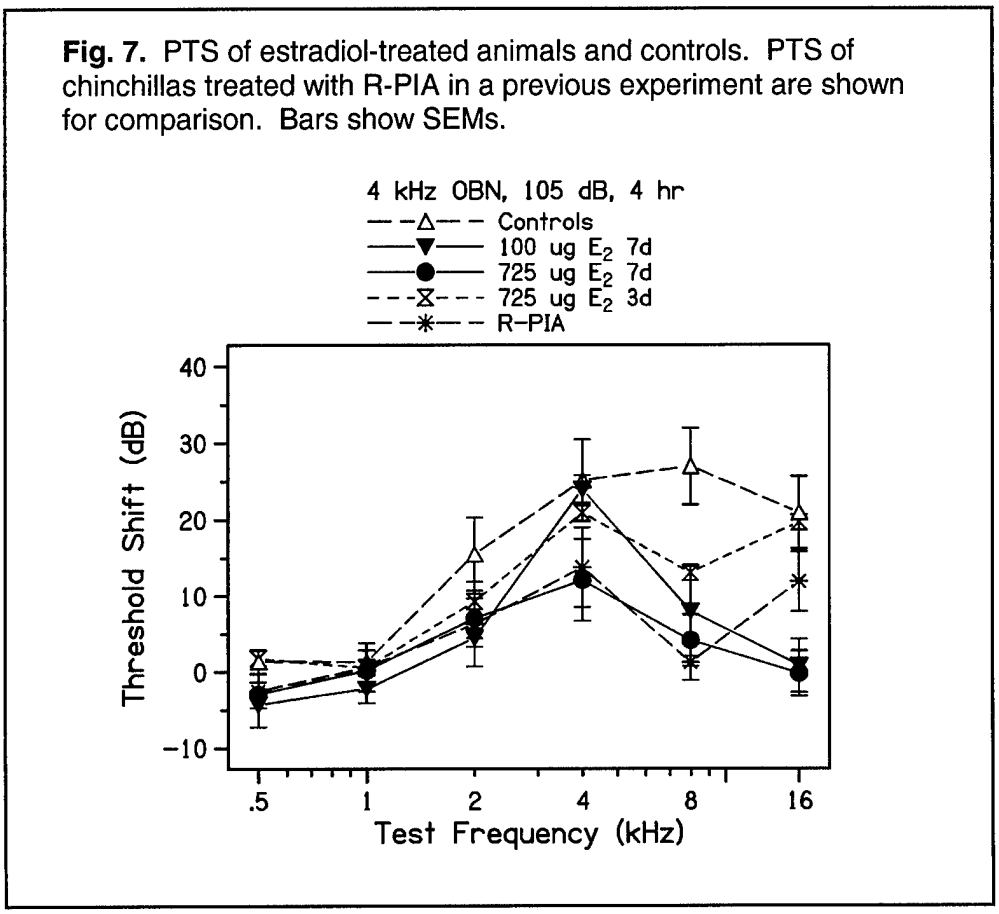

Fig. 7. PTS of estradiol-treated animals and controls. PTS of chinchillas treated with R-PIA in a previous experiment are shown for comparison. Bars show SEMs.

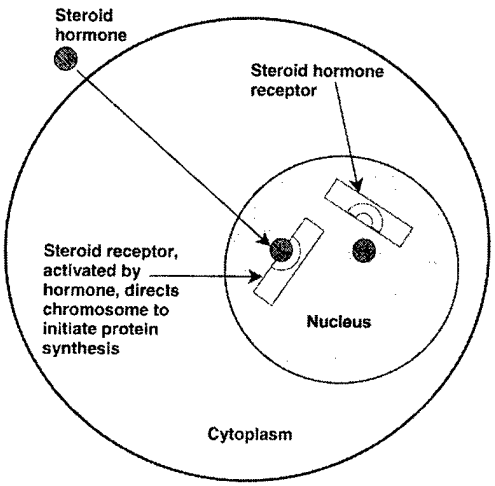

Fig. 8. Schematic illustrating the genomic effects of estradiol. Estradiol binds to an appropriate receptor in the cell nucleus and activates specific genes on nuclear chromosomes. Gene activation results in the synthesis of specific proteins in the cytoplasm.

One interesting finding from the two estradiol experiments was that there were no remarkable differences in hair cell loss between treated animals and controls, despite dramatic differences in PTS. It is not unusual to see a lack of correspondence between hair cell loss and hearing loss in noise-exposure experiments (Hamernik et al., 1989; Boettcher et al., 1992; McFadden et al., 1997). However, the dissociation between threshold shifts and hair cell loss in the estradiol experiments could be interpreted as evidence that estradiol is acting at the level of the stria vascularis (e.g., marginal cells or vascular tissue) rather than the organ of Corti. This interpretation is consistent with previous findings, and with known actions of estradiol.

Like R-PIA, steroid hormones can act through many different routes, each of which could be important for modulating susceptibility to NIHL. Estradiol can act through slow genomic pathways, or rapid non-genomic pathways. Numerous studies have established that estradiol can potentiate GABA-mediated inhibition in the central nervous system, modulate neuronal activity, trigger acetylcholine (ACh) synthesis (note that ACh is the major neurotransmitter of olivocochlear efferents), act directly as a potent antioxidant, and influence the bioactivity of other antioxidants and blood flow promoters such as nitric oxide (Arnal et al., 1996; Ayres et al., 1996; Behl et al., 1995; Chadwick and Widdows, 1990; Goodman et al., 1996; Romer et al., 1997; Ruiz-Larrea et al., 1994). Given the recently identified role of ROS and antioxidants in the etiology NIHL, coupled with our limited evidence that estradiol is acting through genomic pathways, a reasonable hypothesis is that estradiol exerts its protective effects by promoting the synthesis of endogenous antioxidant enzymes in the cochlea. This hypothesis can be tested

directly in future experiments by measuring levels of various antioxidants in the cochlea after animals have been treated with estradiol and exposed to noise.

2.7. Experiments with progesterone pre-treatment—progesterone may exacerbate NIHL

Two experiments were conducted to investigate the effects of progesterone (4-Pregnene-3,20-dione, Sigma Chemicals) on noise-induced threshold shifts and hair cell loss. Chinchillas received a subcutaneous injection of progesterone each day for 5 days, for a total dose of 5 mg. After pre-testing, treated animals and control animals were exposed to impulse noise at 150 dB pSPL (Experiment I), or 4 kHz octave band noise at 105 dB SPL for 4 hr (Experiment II). With the single dosing protocol and the relatively small numbers of animals tested, it is difficult to draw conclusions about the influence of progesterone on NIHL. However, our preliminary results suggest that unlike estradiol, progesterone either has no effect on NIHL, or exacerbates damage. Figure 9 illustrates the effects of progesterone on PTS caused by 4 kHz OBN. Animals treated with progesterone had slightly greater hearing losses than controls.

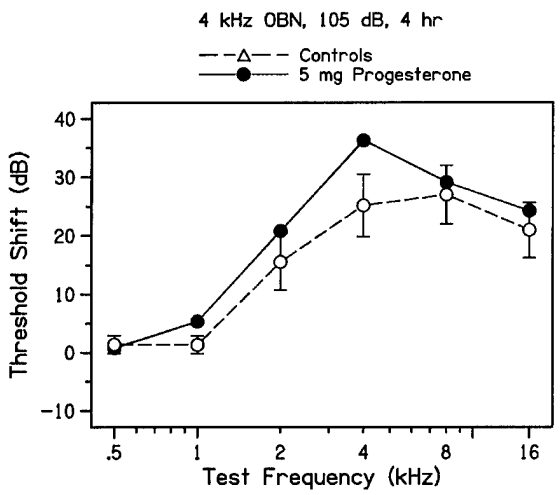

Fig. 9. PTS of controls and animals treated for 5 days with progesterone. Bars show SEMs.

3. Problems

Problems encountered during the third year of the project prompted us to apply for a no-cost extension of the grant. Problems included difficulty attaining and housing sufficient numbers of chinchillas for experiments, poor health of many animals obtained from a new breeder, changes in personnel, and the need to develop new techniques for assaying hormone levels from serum samples. The one-year no-cost extension we received enabled us to finish all projects as originally outlined in our Statement of Work.

III. Key Research Accomplishments

1. There are significant differences between females and males in basic auditory sensitivity.

- Chinchillas exhibit small, but consistent sex differences in auditory sensitivity, with females showing slightly better thresholds at high frequencies, but slightly worse thresholds at low frequencies than males. This pattern matches the pattern described for humans.

2. There are fundamental differences between females and males in the response of the ear to high-level noise.

- Chinchillas exhibit sex differences in their susceptibility to hearing loss caused by high-level impulse noise (simulated M16 rifle fire). Female chinchillas consistently develop significantly less hearing loss at low frequencies, but more hearing loss at high frequencies than males.

3. Low-frequency noise is an effective sound conditioning stimulus, but helicopter noise is not.

- Chinchillas exposed to helicopter noise at 90 or 112 dB SPL for 5 or 10 days (1.5 hr each day) were not protected from PTS or hair cell loss caused by subsequent exposure to simulated M16 rifle fire.
- Exposure to helicopter noise at 90 dB SPL (i.e., a level that might be experienced by a soldier wearing hearing protectors while riding in the passenger area of the helicopter) neither increased nor decreased susceptibility to hearing loss caused by a subsequent exposure to simulated M16 rifle fire.
- Exposure to 112 dB SPL helicopter noise (the level experienced by a soldier sitting in the passenger cabin without hearing protectors) exacerbated hearing loss and cochlear damage from M16 rifle fire.
- A 5-day conditioning protocol utilizing a "standard" conditioning noise (0.5 kHz octave band noise at 90-95 dB SPL) reduced hearing loss from simulated M16 rifle fire for both female and male chinchillas. Female chinchillas showed only slightly more benefit than males under these exposure conditions.
- Although significant protection was provided by the 5-day "standard" conditioning protocol, the magnitude of protection was less than that obtained after 10 days of sound conditioning with the same noise.

4. Pharmacological manipulations can protect the ear from NIHL.

- When applied to the round window membrane of the inner ear, R-phenylisopropyladenosine (R-PIA) provided significant protection from NIHL.
- The magnitude of protection provided by R-PIA was similar for female and male chinchillas, despite significant differences in PTS magnitude between the sexes.

5. Estradiol levels influence PTS but not cochlear hair cell loss.

- Serum estradiol levels of female and male chinchillas vary on a continuum comparable to humans, and could contribute to individual differences in susceptibility to NIHL.
- The protective effects of estradiol on noise-induced hearing loss were demonstrated in two experiments that utilized different noise exposure conditions (continuous octave band noise centered at 4 kHz, and impulse noise simulating M16 rifle fire) and a variety of estradiol doses. Thus, the protective effects of estradiol are robust and reliable.

- Estradiol treatment for 7 days (725 μg total) provided more protection than R-PIA. Unlike R-PIA, the protective effects of estradiol were achieved with simple systemic treatment rather than invasive surgery to expose the round window membrane.
- Although estradiol treatment provides significant protection from PTS, it has little effect on cochlear hair cell loss. Thus, estradiol may be exerting its effects on the stria vascularis rather than the organ of Corti.
- Evidence suggests that estradiol acts primarily through genomic mechanisms, perhaps by initiating the synthesis of antioxidant enzymes in the cochlea.

6. Progesterone was not protective under limited experimental conditions.

- Unlike estradiol, progesterone did not protect chinchillas from NIHL caused by impulse noise or continuous noise. Because only one dosing protocol was used, however, it is premature to draw conclusions about the effects of progesterone on susceptibility to NIHL.

IV. Reportable Outcomes

A. Abstracts, Presentations, Review Articles and Book Chapters (in chronological order):

1. McFadden, S.L., Zheng, X.Y., Ding, D.L., and Henderson, D. (1999) Differences between female and male chinchillas in susceptibility to noise-induced hearing loss. *Assoc. Res. Otolaryngol. Abstr.* 610, 155.
2. McFadden, S.L. Overview of research on steroid hormones and noise-induced hearing loss. *Lake Ontario Hearing Meeting*, Syracuse University, June 15, 1999.
3. Henderson, D., McFadden, S.L., Liu, C.C., Hight, N., and Zheng, X.Y. (1999) The role of antioxidants in protection from impulse noise. In: D. Henderson, R.J. Salvi, A. Quaranta, S.L. McFadden, and R.F. Burkard (eds.), Annals of the New York Academy of Sciences, Vol. 884, Ototoxicity: Basic Science and Clinical Applications. NY: New York Academy of Sciences, pp. 368-380.
4. Henderson, D., McFadden, S.L., Zheng, X.Y., Kopke, R., and Hight, N. (1999) Intervention possibilities for noise induced hearing loss. In: D. Prasher and B. Canlon (Eds.), Cochlear Pharmacology and Noise Trauma. London: NRN Publishers, pp. 85-95.
5. McFadden, S.L., and Henderson, D. (1999) Recent advances in understanding and preventing noise-induced hearing loss. *Current Opinion in Otolaryngology*, 7, 266-273.
6. Lockwood, D., McFadden, S.L., Jiang, H., and Rosenberg, L. (2000) Systemic treatment with estradiol reduces noise-induced hearing loss in the chinchilla. *Assoc. Res. Otolaryngol. Abstr.* 167, 48.

*B. Publications in Peer-Reviewed Journals (in chronological order; *denotes article is included in Appendix I):*

*1. McFadden, S.L., Henselman, L.W., and Zheng, X.Y. (1999). Sex differences in auditory sensitivity of chinchillas before and after exposure to impulse noise. *Ear & Hearing* 20, 164-174.

*2. Henderson, D., Hu, B.H., McFadden, S.L., and Zheng, X.Y. (1999) Evidence of a common pathway in noise-induced hearing loss and carboplatin ototoxicity. *Noise and Health* 5, 53-69.

*3. Hu, B.H., McFadden, S.L., Salvi, R.J., and Henderson, D. (1999) Intracochlear infusion of buthionine sulfoximine potentiates carboplatin ototoxicity in the chinchilla. *Hearing Research* 128, 125-134.

*4. McFadden, S.L., Zheng, X.Y., and Ding, D.L. (2000) Conditioning-induced protection from impulse noise in female and male chinchillas. *Journal of the Acoustical Society of America* 107, 2162-2168.
5. McFadden, S.L., Zheng, X.Y., and Ding, D.L. Sex differences in threshold shifts and hair cell loss in chinchillas exposed to simulated military noises (manuscript in preparation; to be submitted to *Noise & Health*).
6. McFadden, S.L., Lockwood, D., Rosenburg, L., and Jiang, H. Systemic treatment with 17-β estradiol reduces NIHL in chinchillas (manuscript in preparation, to be submitted to *Hearing Research*).

C. Degrees Obtained that were Supported by this Award:

Marty Howard, M.S., CCC--currently employed as Audiologist at VA Medical Center, Canandaigua, NY and Rochester VA Hospital, Rochester, NY.

D. List of Personnel Receiving Pay from the Research Effort:

1. Sandra L. McFadden, P.I.
2. Donald Henderson, Co-Director of Center for Hearing and Deafness
3. Carol Altman, Administrative Assistant and Grant Manager
4. Xiangyang Zheng, Research Scientist
5. Haiyan Jiang, Research Scientist
6. Marlene Shero, Research Scientist
7. Marty Howard, Graduate Student
8. Daniel Lockwood, Graduate Student

V. Conclusions

The results from the experiments described here have both theoretical and practical implications. From a theoretical standpoint, our experiments provide a much-needed perspective on the source of sex/gender differences in basic auditory processing and susceptibility to NIHL, as well as a perspective on the role of steroid hormones in the cochlea. When gender differences in NIHL have been observed in humans, it has been customary to attribute the differences to noise-exposure history. The classical argument is that men develop more hearing loss than women because they are exposed to more occupational and/or recreational noise. Our results with chinchillas are the first to show sex differences in NIHL in an animal model where noise exposure history and other confounding factors are controlled. The findings strengthen the hypothesis that some gender differences in humans are due to inherent anatomical or physiological factors, rather than noise-exposure history. One implication is that extra steps must be taken to protect males from low-frequency hearing loss. Further exploration of underlying factors may be useful in reducing susceptibility to low frequency PTS and hair cell loss in males.

From a practical standpoint, we have demonstrated the feasibility of using sound conditioning and pharmacological agents to reduce NIHL. Despite inherent sex differences in susceptibility to NIHL, both females and males benefited from a standard low-frequency sound conditioning protocol. Furthermore, the magnitude of protection (reduction of PTS and OHC loss) from sound conditioning was similar between the sexes. This is further evidence that sound conditioning targets OHCs. We have also shown a clear link between levels of endogenous

steroid hormones and susceptibility to NIHL. Based on our findings, it is reasonable to hypothesize that individuals with high levels of estrogen will be less susceptible to NIHL than individuals with low levels of estrogens. The results provide a strong basis for interpreting studies with human females that have shown cyclic fluctuations in both hearing sensitivity and susceptibility to NIHL. For women, it is likely that susceptibility to NIHL is lowest when estradiol peaks in the menstrual cycle, and highest during the progesterone surge. A feasible extension of our experiments is to develop estrogenic compounds that protect the ear without producing unwanted side effects, and to test the otoprotective potential of such compounds (e.g., tamoxifen) that are already available. A tremendous advantage of this approach is that it does not require invasive surgery to target the inner ear.

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List of Appendices

Appendix I. Publications acknowledging support by the USAMRMC.

Appendix II. S.L. McFadden Curriculum Vitae

APPENDIX IA

Sex Differences in Auditory Sensitivity of Chinchillas Before and After Exposure to Impulse Noise

Sandra L. McFadden, Lynn W. Henselman, and Xiang-Y. Zheng

Objective: **To determine if chinchillas exhibit sex differences in 1) basic auditory sensitivity and 2) susceptibility to cochlear damage and hearing loss from high-level impulse noise.**

Design: **The auditory sensitivity of 73 chinchillas was assessed by measuring evoked potentials from electrodes implanted in the inferior colliculus (IC-EVPs) and cubic ($2f_1$-f_2) distortion product otoacoustic emissions (CDPs). A subgroup of 16 chinchillas were retested after exposure to simulated M16 rifle fire (150 dB pSPL impulse noise). Thresholds and postexposure temporary and permanent threshold shifts were compared as a function of sex and frequency using analysis of variance procedures. Cochleograms, showing the percent of hair cells missing as a function of location on the basilar membrane, were constructed to show inner hair cell (IHC) and outer hair cell (OHC) losses for each group.**

Results: **Female chinchillas had slightly lower high-frequency thresholds, and slightly higher low-frequency thresholds than male chinchillas, but similar IC-EVP and CDP amplitude functions. Significant sex differences were observed after exposure to high-level impulse noise. Overall, female chinchillas developed approximately 10 dB more high-frequency hearing loss, but approximately 5 dB less low-frequency hearing loss than males. Hair cell losses, particularly IHC losses, were substantially less for females as compared with males.**

Conclusions: **The results point to close similarities between chinchillas and humans with regard to sex/gender differences in basic auditory sensitivity before noise exposure, suggesting that the chinchilla may be a good model for exploring the anatomical and physiological bases of these differences. In addition, the results show significant sex differences in the physiological and anatomical response of the chinchilla cochlea to high-level noise. Similar differences in humans could have important implications with regard to military assignments and hearing conservation programs.**

(Ear & Hearing 1999;20;164–174)

Center for Hearing and Deafness (S.L.M., X.Y.Z.), University of Buffalo, Buffalo, New York; and Office of the Inspector General (L.W.H.), Department of Defense, Arlington, Virginia.

Noise-induced hearing loss (NIHL) is a major occupational hazard for military personnel because of the types and levels of noise encountered in training and combat situations (Dancer & Franke, 1986; Henselman, Henderson, Shadoan, Subramaniam, Saunders, & Ohlin, 1995; Henselman, Henderson, Subramaniam, & Sallustio, 1994). Damage to the cochlea can be caused by a variety of acoustic events, ranging from prolonged exposure to continuous noises that cause metabolic and biochemical changes in the cochlea, to relatively brief exposures to high-level impact and impulse noises such as gunfire, cannon fire and explosions, that can produce direct mechanical damage as well (Dancer & Franke, 1986; Henderson, Hamernik, & Sitler, 1974; Henderson, Spongr, Subramaniam, & Campo, 1994). A recognition of the serious consequences of NIHL led the U.S. Air Force to develop the first hearing conservation program (HCP) in 1948. The U.S. Navy and U.S. Army developed similar HCPs in 1955 and 1956, respectively (Henselman et al., 1995). Since their inception, military HCPs have served to increase awareness of the damaging effects of high-level noise exposure. They have also served to reduce the incidence and magnitude of NIHL in military personnel, primarily by mandating the use of personal protection devices such as sound-attenuating ear plugs or earmuffs in high-noise situations. However, NIHL remains a serious problem for military personnel who are exposed to loud noises during training and combat situations in which personal protection devices are either unavailable, impractical or dangerous to use, improperly fitted or worn, or inadequately designed to protect the ear from damage (Dancer, Buck, Parmentier, & Hamery, 1998).

As women become more fully integrated into a variety of military occupational specialties, many will be placed at risk for developing NIHL. It is critical, therefore, that we understand the specific relationship between noise exposure and hearing loss in women, so that appropriate measures for preventing NIHL can be developed and implemented.

Previous studies (Chung, Mason, Gannon, & Willson, 1983; Corso, 1963; Pearson et al., 1995; Ward,

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1966) have reported small differences (generally less than 3 dB) between males and females in auditory sensitivity (i.e., thresholds for detecting pure tones under quiet listening conditions). In general, females tend to have slightly better pure-tone thresholds than males at frequencies above 1 to 2 kHz, whereas males may have slightly better thresholds below 1 to 2 kHz. Small, but consistent gender differences* have also been reported in susceptibility to temporary threshold shifts (TTS) caused by exposure to continuous tones or noise (Axelsson & Lindgren, 1981; Dengerink, Dengerink, Swanson, Thompson, & Chermak, 1984; Petiot & Parrot, 1984; Ward, 1966). In general, experimental studies of TTS in humans have found that males exhibit more TTS than females from low-frequency exposures (below 2 kHz), whereas females exhibit more TTS than males from high-frequency exposures (above 2 kHz). In an early investigation of gender differences in susceptibility to TTS produced by high intensity tones and noise, Ward (1966) conducted 17 experiments with 24 male and 25 female adults. Females showed approximately 30% less TTS than males when the exposure frequency was below 1 kHz, but approximately 30% more TTS when the exposure frequency was above 2 kHz.

The above studies examined TTS rather than the more important issue of permanent threshold shift (PTS) because it is not ethical to intentionally induce PTS in human subjects. Most of what little we know about gender differences in PTS comes from retrospective studies of workers exposed to noise in industrial settings (Berger, Royster, & Thomas, 1978; Gallo & Glorig, 1964). Under these conditions, which typically involve exposure to low-frequency continuous noises, males tend to develop much more hearing loss than females. Both Berger et al. (1964) and Gallo and Glorig (1964) found approximately 20 dB more PTS in males than in females after 9 yr of industrial noise exposure. These results are consistent with the gender differences observed in Ward's (1966) studies of TTS. However, there are no comparable studies of gender differences in PTS produced by exposures to high-level impulse noises that are typically found in military environments. A finding of gender differences in susceptibility to NIHL could have important implications for military assignments and HCPs.

The present study was conducted to determine whether there are systematic differences between female and male chinchillas in 1) basic auditory function, as assessed by inferior colliculus evoked potentials (IC-EVPs) and cubic ($2f_1$-f_2) distortion product otoacoustic emissions (CDPs), and 2) their susceptibility to high-level impulse noise. Basic auditory function was assessed in a relatively large group of chinchillas (N = 73). Susceptibility to impulse noise was examined in a subgroup of these animals (N = 16). Findings from the chinchilla may shed light on gender differences in susceptibility to impact/impulse noise, and offer insights into the anatomical and physiological mechanisms contributing to documented gender differences in humans.

* The term "gender differences" will be used to refer to male/female differences in humans, and the term "sex differences" will be used to refer to male/female differences in chinchillas and other nonhuman species.

METHODS

All procedures described here were reviewed and approved by the University of Buffalo Animal Care and Use Committee, and conformed to federal guidelines for the humane treatment of laboratory animals.

Subjects

A total of 73 chinchillas (*Chinchilla laniger*; 37 female, 36 male) between 1 and 3 yr of age were used. A subgroup of animals (eight female, eight male) was exposed to impulse noise, and their thresholds were measured at various times after exposure (see below). The chinchilla was used for these studies because it 1) is relatively immune to middle ear infections and diseases that affect hearing (Clark, 1984); 2) has a relatively long life span (12 to 20 yr) with minor age-related cochlear pathology and hearing loss before 8 to 10 yr of age (Bohne, Gruner, & Harding, 1990; McFadden, Campo, Quaranta, & Henderson, 1997); and 3) reacts predictably to anesthesia and tolerates surgery well. Most importantly, the chinchilla has a range of hearing that is more similar to that of humans than most other laboratory animals, particularly in the low frequencies (Heffner & Heffner, 1991; Miller, 1970), which enhances its suitability as a model for studying NIHL (McFadden, Campo, Ding, & Quaranta, 1998). With regard to size, Clark (1984) states that female chinchillas tend to be larger than males. In a small group of our chinchillas (eight female, eight male) for which reliable weights were available, weight differences were minor, but favored females. The average weight of females was 572.2 g (SD = 73.7), versus 563.9 g (SD = 70.0) for males.

Surgical Preparation

Each animal was deeply anesthetized with an intramuscular injection of ketamine hydrochloride (60 mg/kg) and acepromazine (0.5 mg/kg). Chronic recording electrodes were implanted in the left and/or right inferior colliculus (IC), and in the ros-

tral cranium (McFadden et al., 1997). Thirteen animals were implanted unilaterally; all others were implanted bilaterally. A small hole was drilled in the dorsal cranium overlying the IC, and a recording electrode mounted on a stereotaxic device was advanced through the IC while the surgeon monitored sound-evoked electrical activity on audio and video monitors. When the electrode had been advanced to a depth that produced clear, large amplitude EVPs, it was cemented to the skull with cyanoacrylic adhesive and dental cement. A short electrode was implanted in the rostral cranium to serve as the common lead for IC-EVP recording. Because the electrodes remain fixed in position, variability associated with changes in electrode placement between tests is eliminated. In addition, the signal to noise ratio is much better with implanted electrodes than with more conventional scalp electrodes, so that thresholds can be determined with greater precision. IC-EVPs recorded from electrodes implanted in this manner yield thresholds that are very close to behavioral thresholds measured in the same animals (Henderson, Hamernik, Salvi, & Ahroon, 1983), and about 15 to 20 dB lower than threshold estimates obtained using subcutaneous electrodes in the same animals (Murphy & Themann, Reference Note 1). After surgery, the animals recovered in a quiet animal colony for at least 1 wk before testing.

Measures of Auditory Function

The auditory sensitivity of each animal was assessed by measuring IC-EVPs. CDPs were also obtained from most animals. All testing was conducted in a sound-attenuating booth (Industrial Acoustics Corp. 400) lined with sound-absorbing foam panels. The awake chinchilla was placed in a custom-designed tube (Snyder & Salvi, 1994) that held its head at a constant orientation within the calibrated sound field.

Stimuli for IC-EVP testing consisted of 10 msec tones (2 msec Blackman rise/fall ramp, alternating phase) at octave intervals from 0.5 to 16 kHz, presented at a rate of 20/sec. Stimuli were generated digitally (93 kHz sampling rate) by a 16 bit D/A converter on a digital signal processing (DSP) board (TMS320C25) in a personal computer and routed through computer-controlled attenuators and impedance matching transformers to a loudspeaker (Realistic 401197) located on the side of the test ear, at a distance of approximately 9 cm from the animal's pinna. The nontest ear was plugged with a foam insert earplug, providing approximately 20 to 40 dB attenuation in addition to the attenuation produced by the animal's head and body obstructing the propagation of sound to the opposite ear. Electrical activity from the IC electrode contralateral to the test ear was amplified (20,000×), filtered (10 to 3000 Hz), and converted to digital signals (50 kHz sampling rate) by an A/D converter on a separate computer DSP board. Stimuli were presented in ascending order of frequency and intensity. Fifty or 100 trials were computer averaged at each stimulus level and the level was incremented in 5 dB steps. Figure 1 illustrates IC-EVP waveforms (raw data) obtained from a normal chinchilla.

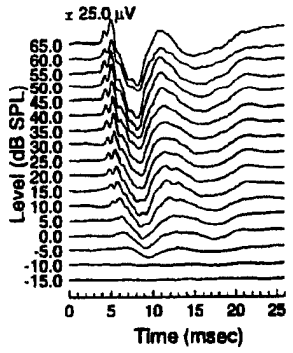

Figure 1. Typical inferior colliculus evoked potential waveforms obtained from a normal chinchilla. Stimulus frequency was 8 kHz. Threshold is −7.5 dB SPL.

Stored waveforms were analyzed visually to determine thresholds. Threshold (dB SPL re: 20 µPa) was defined as the mid-point between the level at which there was a clear deflection in the waveform and the next lower level at which there was none. For example, if there was a clear response at −5 dB SPL and none at −10 dB SPL, the threshold was recorded as −7.5 dB SPL (see Fig. 1).

CDP measurements were made using a system designed in our lab that utilizes three DSP boards housed in a personal computer, insert earphones (Etymotic ER-2), a low noise probe microphone (Etymotic ER-10B), and custom-built attenuators and amplifiers. One DSP board processes microphone output while the other two generate digital signals (primary tones, f_1 and f_2). The primary tones were generated at a sampling rate of 93 kHz and output through 16 bit D/A converters. The microphone output was routed to a 16 bit A/D converter and digitized at a rate of 31 kHz. A Blackman windowing function was applied to the incoming data stream, and a partial discrete Fourier transform was computed. Frequency components corresponding to the two primary frequencies, the cubic distortion product ($2f_1$-f_2), and the noise floor (f_n = 0.7 CDP) were computed. A calibration measurement preceded each input/output (I/O) function, in which the primary tones were presented at an attenuation of 20 dB and the output levels at the primary frequencies were measured and used as reference levels. I/O

functions were collected for primary tones (f_2 = 1.2, 2.4, 3.6, 4.8, 7.2, 9.6, and 12 kHz; f_2/f_1 = 1.2) from 0 to 70 to 80 dB SPL in 5 dB steps. CDP tests followed IC-EVP testing.

Noise Exposures and Acoustic Calibration

The impulse noise was a modified Friedlander wave (0.8 msec A-duration), with a time-amplitude profile simulating impulses created by 5.56 mm rounds fired from a U.S. Army M16A1 rifle (Danielson, Henderson, Gratton, Bianchi, & Salvi, 1991). The digital signal was converted to analog by a D/A converter on a DSP board, attenuated (HP 350D), amplified (NAD 2200), and routed in parallel to two compression drivers (JBL 2446) coupled to sound delivery tubes (5 cm diameter × 20 cm). The ends of the sound delivery tubes were cut at 45° angles to broaden the range of the tube's resonance (Danielson et al., 1991). The drivers faced each other, with the sound delivery tubes separated by 10 cm. Acoustic foam wedges surrounded the drivers to minimize reverberation. An animal was placed in a restraint tube in the 10 cm space between the opposing sound tubes, and 50 pairs of impulses (100 total) were delivered simultaneously to both ears. Impulses in each pair were spaced 50 msec apart, and there was a 1000 msec interval between the onset of each pair (Henselman et al., 1994). The duration of the exposure was therefore less than one minute for each animal.

All exposures were conducted in a 1.8 m × 2.0 m sound booth (Acoustic Systems), where animals were exposed individually. A 1/8" microphone (Bruel & Kjaer Model 4138) was used for acoustic calibration of the impulse noise. The voltage corresponding to a 114 dB tone produced by a pistonphone coupled to the microphone was determined, and used to calculate the desired voltage for a 150 dB peak SPL signal. The attenuation of a manual attenuator (Hewlett Packard 350D) was adjusted to produce the desired signal voltage.

Test Schedule after Noise Exposure

IC-EVPs and CDPs were measured from impulse noise-exposed animals at 15 minutes, 24 hr, and 5 days postexposure to monitor TTS, and after 25 to 35 days recovery from exposure to determine PTS. Before exposure, each animal was tested three times, and the average of the three measurements was used as the stable baseline estimate of sensitivity. Threshold shifts (TSs) of each animal were calculated by subtracting mean pre-exposure IC-EVP thresholds from postexposure thresholds. After 25 to 35 days recovery from high-level exposure, IC-EVPs and CDPs were measured on three separate occasions and averaged to calculate PTS at each frequency.

Cochlear Histology

At the end of testing, chinchillas were deeply anesthetized with sodium pentobarbital (Somlethal, 100 mg/kg i.p.) and decapitated. The cochleas were quickly removed and perfused through the oval window with a succinate dehydrogenase staining solution (2.5 mL, 0.2 M sodium succinate, 2.5 mL, 0.2 M phosphate buffer, pH 7.6, and 5 mL, 0.1% tetranitro blue tetrazolium). Cochleas were then incubated in the succinate dehydrogenase staining solution for 45 minutes at 37° C, postfixed with 10% formalin, and stored in fixative. Stained cochleas were dissected from the apex to the base, mounted in sections in glycerin on microscope slides, coverslipped, and examined using light microscopy (400× magnification). Percent hair cells missing was referenced to our lab standards based on average hair cell counts from nine cochleas of young (<1 yr old), healthy chinchillas.

Data Analyses

Data were obtained from both ears of 37 animals (19 male, 18 female) and from a single ear of 36 animals, so that the final sample for data analysis consisted of 110 ears (55 male, 55 female). Noise-exposure data were obtained from 28 ears (15 male, 13 female). Data analyses were geared toward answering the following questions: 1) Are there significant sex differences in auditory sensitivity, IC-EVP amplitudes, or CDP I/O functions? 2) Are there sex differences in TTS and/or PTS caused by exposure to simulated M16 rifle fire? Analyses of variance (ANOVAs) were used to assess differences between means. The dependent variables were IC-EVP thresholds and IC-EVP threshold shifts (TSs) at various times after noise exposure. Independent variables were Sex (a between-subjects factor), Frequency and Time of Assessment (within-subjects factors). Significant main effects and interactions involving Sex were analyzed further using 1-way ANOVAs or independent Student *t*-tests. Within a group, changes as a function of time or frequency were assessed using paired *t*-tests. Mean IC-EVP and CDP amplitude functions for females and males were compared by calculating the 95% confidence interval for the difference between the means. All statistical tests were evaluated using a 0.05 criterion of significance.

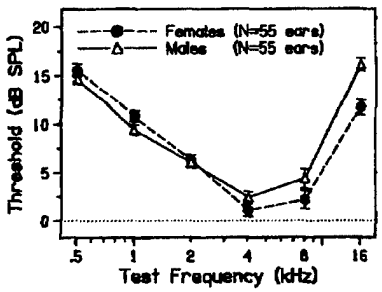

Figure 2. Pre-exposure thresholds of female (solid circles) and male (open triangles) chinchillas. Differences between females and males were statistically significant at 16 kHz only. Bars represent standard errors of the means.

RESULTS

Basic Auditory Sensitivity of Female and Male Chinchillas

IC-EVP Thresholds • Thresholds of female and male chinchillas are shown in Figure 2. As a group, male chinchillas have slightly lower thresholds than females at frequencies below 2 kHz, whereas female chinchillas have slightly lower thresholds than males at frequencies above 2 kHz. The differences are generally small, but consistent.

A 2-way mixed ANOVA, with Sex as a between-subjects factor and Frequency as a within-subjects factor, revealed a significant Sex × Frequency interaction, $F(5, 540) = 7.58$; $p < 0.001$. Follow-up analyses indicated that mean threshold at 16 kHz was significantly higher for males than for females (16.15 ± 4.8 dB versus 11.64 ± 5.7 dB), $F(1, 108) = 20.24$; $p < 0.0001$. Thresholds at frequencies below 16 kHz were not significantly different between the two sexes.

IC-EVP and CDP Amplitude Functions • Mean IC-EVP I/O functions at 0.5, 1, 2, 4, 8, and 16 kHz are shown in Figure 3. The thin lines represent means for female chinchillas, and the hatched regions surrounding them represent the 95% confidence intervals. The thick lines represent the means for male chinchillas. It is apparent from Figure 3 that there were no significant sex differences in mean I/O functions despite slight differences in IC-EVP thresholds (see Fig. 2).

Similarly, there were no meaningful differences between male and female chinchillas in their CDP I/O functions (Fig. 4). The thin lines in Figure 4 represent means for females, and the hatched regions surrounding them represent the 95% confidence intervals. The thick lines represent means for males. The CDP frequency is indicated above each panel. CDP I/O functions were very similar for males and females, with thresholds around 20 to 30 dB SPL at all frequencies, and amplitudes increasing monotonically over the entire range of input

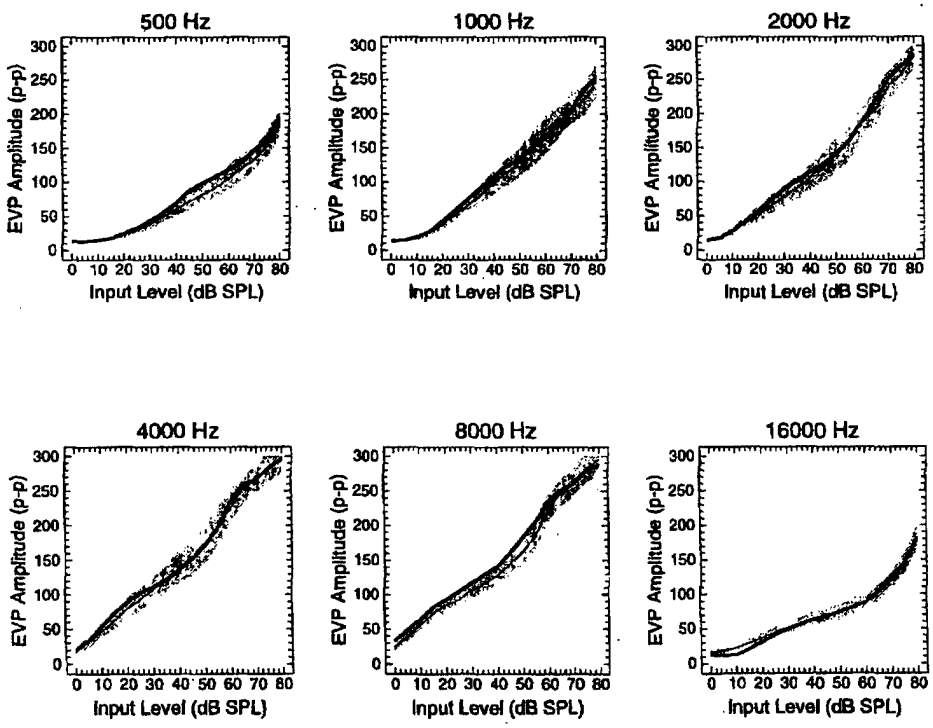

Figure 3. Pre-exposure inferior colliculus evoked potential input/output functions for female (thin line) and male (thick lines) chinchillas. Hatched regions represent the 95% confidence interval for the difference between means. EVP = evoked potential.

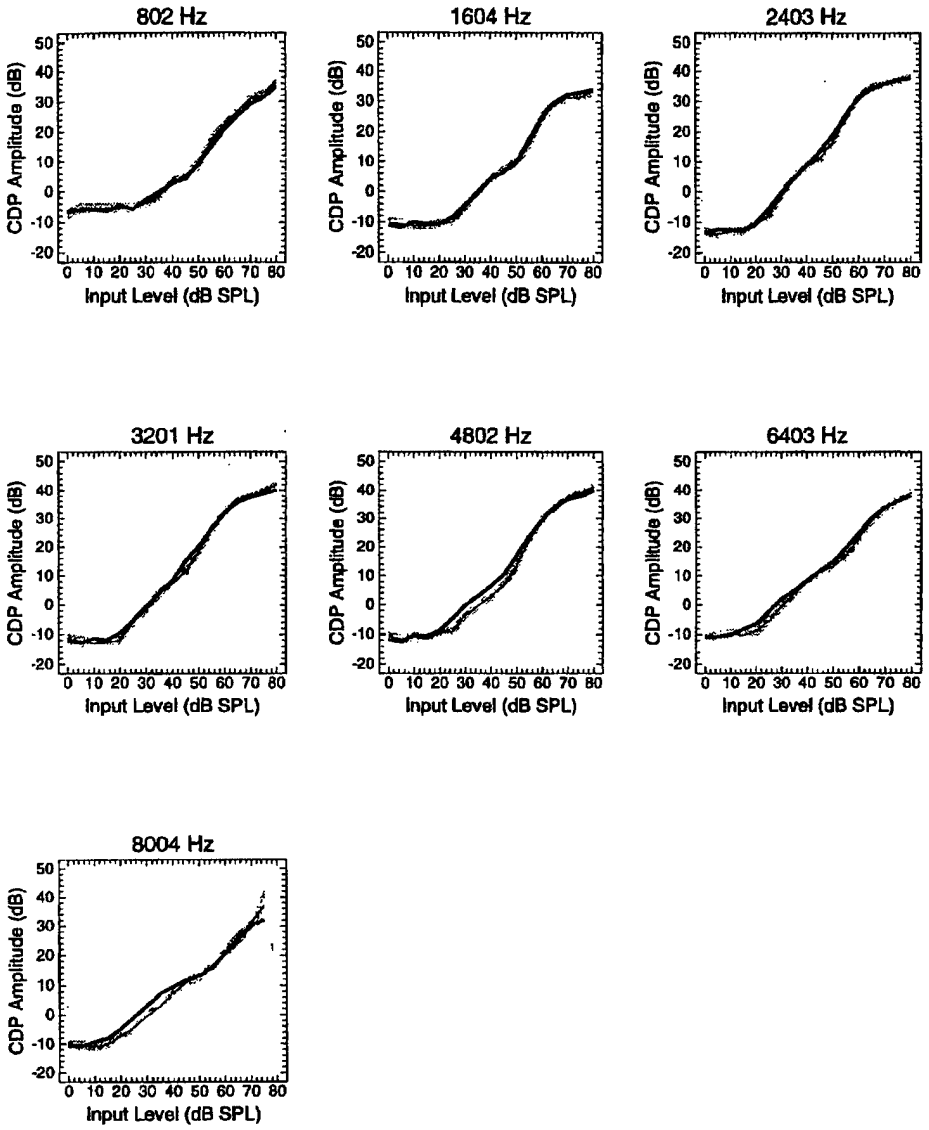

Figure 4. Pre-exposure cubic ($2f_1$-f_2) distortion product otoacoustic emission (CDP) input/output functions for female (thin line) and male (thick lines) chinchillas. Hatched regions represent the 95% confidence interval for the difference between means. The parameter above each panel indicates the CDP frequency ($2f_1$-f_2).

levels. Overall, the results indicate that there are no meaningful sex differences in amplitudes of either IC-EVPs or CDPs before noise exposure, despite small differences in thresholds (Fig. 2).

Sex Differences in Hearing Loss from Simulated M16 Rifle Fire

Pre-Exposure Thresholds • Pre-exposure IC-EVP thresholds for the subset of animals exposed to noise are shown in Figure 5. Although females exhibited slightly lower thresholds than males at several frequencies, particularly at 8 and 16 kHz, a 2-way (Sex × Frequency) mixed ANOVA did not detect significant differences between the sexes.

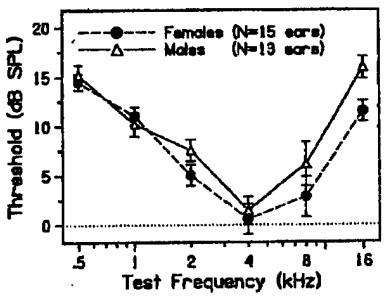

Figure 5. Pre-exposure thresholds of the female (solid circles) and male (open triangles) chinchillas that were subsequently exposed to 150 dB pSPL impulse noise. Bars represent standard errors of the means.

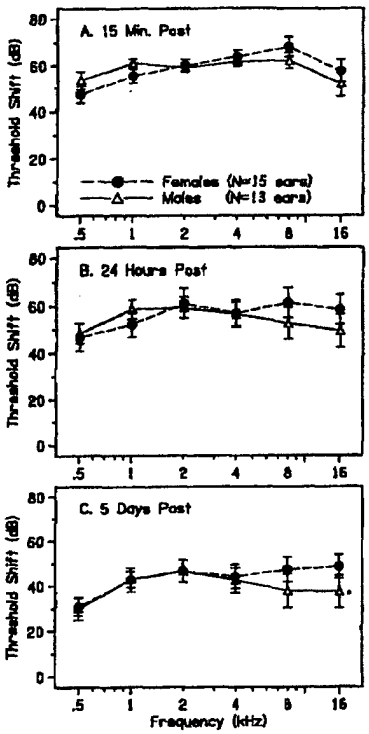

Figure 6. Threshold shifts of female (solid circles) and male (open triangles) chinchillas at 15 minutes, 24 hr, and 5 days after exposure to 150 dB pSPL impulse noise. Bars represent standard errors of the means.

Postexposure TS • Mean IC-EVP TSs measured at 15 minutes, 24 hr, and 5 days after exposure to 150 dB peak SPL impulse noise are shown in Figure 6. When tested 15 minutes after the high-level exposure, both females and males exhibited significant threshold elevations (47 to 68 dB) at all frequencies (Fig. 6a). Males showed approximately 5 to 6 dB more TS than females at 0.5 and 1 kHz, whereas females showed approximately 6 dB more TS than males at 8 and 16 kHz. A 2-way mixed (Sex × Frequency) ANOVA indicated that there was a significant Sex × Frequency interaction, $F(5, 130) = 2.1$; $p = 0.05$, but no main effect of Sex. Follow-up analyses indicated that TS increased progressively from 0.5 kHz to 8 kHz for females. Differences between successive frequencies were significant from 0.5 kHz to 4 kHz; TS was equivalent at 4 and 8 kHz, then declined significantly between 8 and 16 kHz (all p values < 0.01). In contrast, males showed a much flatter pattern of TS, with statistically equivalent TS at 1, 2, 4, and 8 kHz.

Relatively little threshold recovery occurred between 15 minutes and 24 hr postexposure. Females showed TS decreases of 0 to 7 dB, and males showed TS decreases of 0 to 9 dB. Both females and males showed the greatest recovery (5 to 9 dB) at 4 and 8 kHz. As shown in Figure 6b, TS ranged from approximately 47 dB at 0.5 kHz to 61 dB at 8 kHz. Females exhibited approximately 9 dB more TS than males at 8 and 16 kHz, and approximately 5 dB less TS than males at 1 kHz. A 2-way ANOVA yielded a significant Sex × Frequency interaction, $F(5, 130) = 2.4$; $p = 0.044$. Whereas females had statistically equivalent TS at 2, 4, 8, and 16 kHz and significantly less TS at 0.5 and 1 kHz, males had equivalent TS at 1, 2, 4, and 8 kHz, and significantly less TS at 0.5 and 16 kHz than at intermediate frequencies.

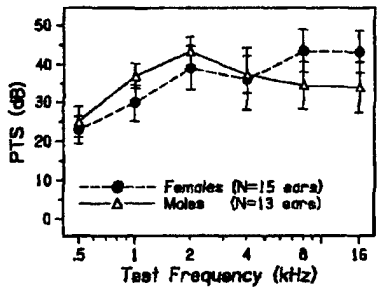

Figure 7. Permanent threshold shifts (PTSs) of female (solid circles) and male (open triangles) chinchillas, measured 30 days after exposure to 150 dB pSPL impulse noise. Bars represent standard errors of the means.

Significant recovery occurred between 1 and 5 days after exposure, with TS decreasing by 9 to 19 dB. Females and males showed equivalent TS at frequencies ≤4 kHz, whereas females exhibited approximately 9 dB and 11 dB more TS than males at 8 and 16 kHz, respectively (Fig. 6c). However, the 2-way (Sex × Frequency) ANOVA did not reveal any significant differences between the sexes at this time.

Mean thresholds improved by 4 to 13 dB between 5 and 30 days postexposure, when permanent hearing loss was assessed (Fig. 7). High-level exposure produced significant PTS at all frequencies for both females and males (paired t-tests; all p values < 0.001). PTS ranged from 23 to 43 dB, with females showing 2 to 7 dB less PTS than males at low frequencies (0.5 to 2 kHz), and approximately 9 dB more PTS at 8 and 16 kHz. A significant Sex × Frequency interaction was obtained, $F(5, 130) = 3.10$; $p = 0.011$. Follow-up analyses indicated that both males and females developed progressively and significantly greater PTS from 0.5 to 2 kHz. However, PTS peaked at 2 kHz for males, and declined significantly at higher frequencies. Females, in contrast, had significantly greater PTS at 8 and 16 kHz than at lower frequencies.

CDP amplitude data are generally consistent with the IC-EVP data. Before noise exposure, CDP I/O functions were similar for males and females, as

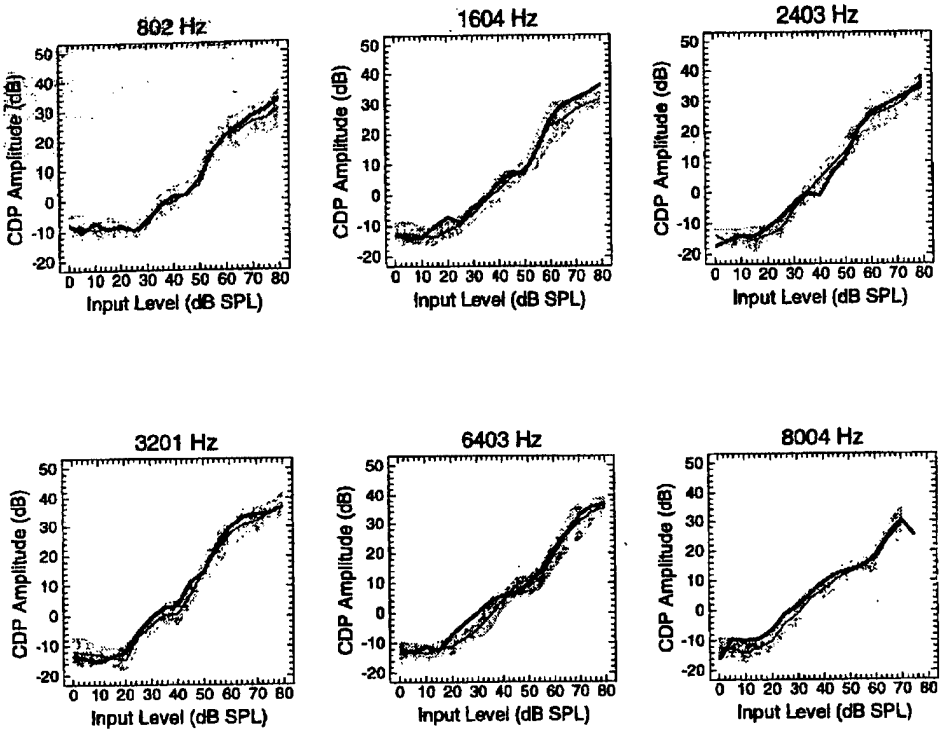

Figure 8. Cubic ($2f_1$-f_2) distortion product otoacoustic emission (CDP) input/output functions for female (thin line) and male (thick lines) chinchillas before noise exposure. Hatched regions represent the 95% confidence interval for the difference between means. The parameter above each panel indicates the CDP frequency ($2f_1$-f_2).

shown in Figure 8. After exposure, however, CDP amplitudes were significantly depressed (Fig. 9). There was a trend for males to have lower amplitude CDPs than females at low frequencies (where males had greater PTS) but higher CDP amplitudes at high frequencies (where males had less PTS).

Hair Cell Losses • Sixteen cochleas (eight female, eight male) were examined for hair cell losses. As shown in Figure 10, outer hair cell (OHC) loss (left panel) exceeded inner hair cell (IHC) loss (right panel), with OHC losses ranging from 70 to 100% in the basal half of the cochlea for both sexes. Males sustained substantially greater IHC and OHC losses than females. IHC losses for males peaked in the 2 to 3 kHz region of the cochlea, with an average loss of approximately 80%. In contrast, average IHC losses for the females did not exceed 30% in any region of the cochlea. OHC losses of females were approximately 20% less than OHC losses of males in the cochlear regions (>1 kHz) where OHC loss occurred.

DISCUSSION

The results indicate that female and male chinchillas differ slightly in their basic auditory sensitivity, with females tending to have lower thresholds at high frequencies and higher thresholds at low frequencies. More importantly, the results point to a fundamental sex difference in the response of the chinchilla cochlea to high-level impulse noise. Female chinchillas sustained more high-frequency hearing loss, less low-frequency hearing loss, and less hair cell loss than males. The reasons for the sex differences observed both before and after noise exposure cannot be determined from this study. However, because the differences were observed in chinchillas, they cannot be attributed to differences in noise exposure history, recreational activities, dietary factors, or other extraneous variables that complicate interpretation of gender differences in humans (Henderson, Subramaniam, & Boettcher, 1993). Therefore, the data from the chinchilla may be particularly useful in interpreting findings from previous studies with humans.

Small but consistent gender differences in auditory sensitivity have been well documented in humans (e.g., Chung et al., 1983; Corso, 1963; Pearson et al., 1995; Ward, 1966). In general, females tend to have slightly lower pure-tone thresholds than males at frequencies above 1 to 2 kHz, whereas males may have slightly lower thresholds below 1 to 2 kHz. Chung et al. (1983) analyzed data from more than 50,000 people and found that the average difference between males and females in hearing sensitivity was 2 to 3.5 dB for test frequencies above 2 kHz, and

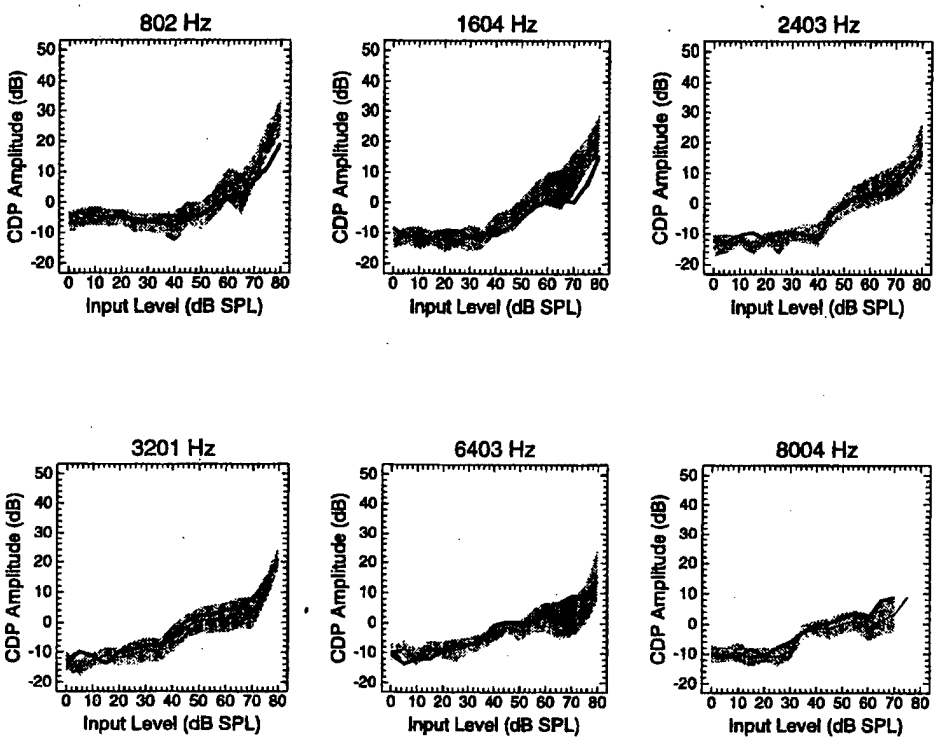

Figure 9. Cubic ($2f_1$-f_2) distortion product otoacoustic emission (CDP) input/output functions for female (thin line) and male (thick lines) chinchillas 30 days after noise exposure. Hatched regions represent the 95% confidence interval for the difference between means. The parameter above each panel indicates the CDP frequency ($2f_1$-f_2).

less than 1 dB for frequencies at or below 2 kHz. Ward (1966) found that thresholds of young adult females were up to 6 dB better than thresholds of young adult males at frequencies above 2.8 kHz. Although differences in auditory sensitivity have sometimes been attributed to gender-related differences in noise exposure history, the current data from chinchillas argue for inherent anatomical and/or physiological differences between the sexes. Recently, Pearson et al. (1995) reported the results of the Baltimore Longitudinal Study of Aging, which tracked thresholds of 681 men and 416 women in low-noise occupations who were screened for otological disorders and NIHL. Their results provide further evidence of small gender differences in thresholds while ruling out occupational noise exposure as the cause for poorer thresholds in men. Women had significantly better thresholds than men at all frequencies above 1 kHz, whereas men had better thresholds at 0.5 kHz, and men and women did not differ at 1 kHz.

Sex/gender differences in both basic sensitivity and in susceptibility to NIHL could arise from differences in the acoustical properties of the outer and middle ears. In a recent study, Hellstrom (1995b) showed a significant relationship between the sound transfer function (STF) of the external ear, ear canal dimensions, and hearing levels in male and female

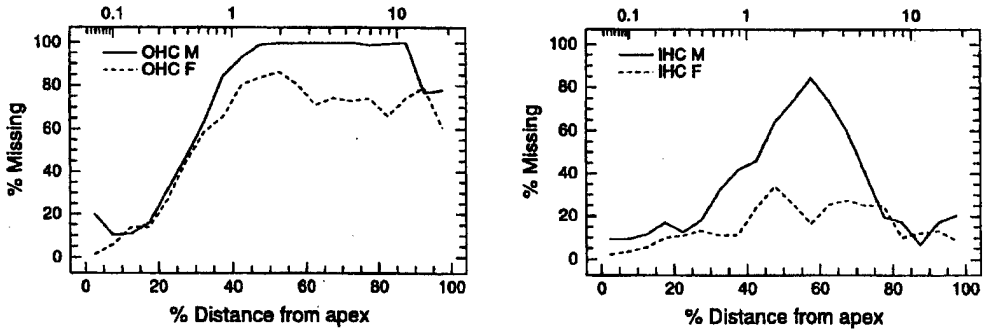

Figure 10. Mean hair cell losses after impulse noise exposure. Left panel: outer hair cell (OHC) loss; right panel: inner hair cell (IHC) loss.

subjects. Females tended to have ear canals that were shorter and smaller in volume than males, resulting in an average STF that was shifted toward higher frequencies. Gender differences in the STF of the external and middle ears would be expected to influence susceptibility to NIHL as well as basic auditory sensitivity (Hellstrom, 1995a, b; Saunders & Tilney, 1982; Tonndorf, 1976).

We are not aware of any published studies of sex differences in ear canal characteristics in nonhuman species. Consequently, the possibility that the sex differences observed in the present study are due to systematic differences in STFs cannot be ruled out. However, several factors suggest that the STF is not the sole basis for sex/gender differences in chinchillas or humans. First, data presented by Saunders and Tilney (1982) show that the chinchilla ear canal STF is a sharply peaked function, with gain increasing from approximately 5 dB SPL at 4.8 kHz to 23 dB at 10 kHz, then dropping to 5 dB around 14 kHz. This STF contrasts with the human ear canal STF, which has a resonant peak between 2 and 4 kHz (Hellstrom, 1995b), yet the pattern of sex/gender differences for chinchillas and humans are quite similar. Hellstrom himself noted that certain aspects of his data were difficult to account for in terms of STF. In particular, there is no obvious reason why subjects with elevated STFs at 4 kHz tended to have lower thresholds at 2 kHz than subjects with elevated STFs at 2 kHz.

A second point to consider is that there are numerous gender differences that are not easily accounted for by the STF. Gender differences have been observed in 1) the upper limit for perceiving binaural beats (Tobias, 1965), with women having a significantly lower cutoff frequency than men (600 versus 800 Hz); 2) the incidence of spontaneous otoacoustic emissions, with women exhibiting them significantly more often than men (Bell, 1992; Bilger, Matthies, Hammel, & DeMorest, 1990; Whitehead, Baker, & Wilson, 1989); and 3) auditory brain stem responses, with women having shorter central conduction times, even after differences in head size are taken into account (Edwards, Squires, Buchwald, & Tanguay, 1983; Patterson, Michalewski, Thompson, Bowman, & Litzelman, 1981; Trune, Mitchell, & Phillips, 1978). Gender differences such as these suggest that factors other than simple acoustics may be involved. Third, studies have reported that hearing sensitivity (Baker & Weiler, 1977; Cox, 1980; Davis & Ahroon, 1982; Miller & Gould, 1967; Swanson & Dengerink, 1988), spontaneous otoacoustic emissions (Bell, 1992; Penner, 1995), auditory brain stem responses (Elkind-Hirsch, Stoner, Stach, & Jerger, 1992), and susceptibility to TTS (Davis & Ahroon, 1982; Dengerink et al., 1984; Petiot & Parrot, 1984) can all fluctuate in monthly cycles in women, or differ between normally cycling women and women taking oral contraceptives. The above considerations suggest that factors other than (or in addition to) the STF may be responsible for sex/gender differences in basic auditory sensitivity and susceptibility to NIHL. Future studies using the chinchilla may help determine the relative importance of anatomical and physiological factors in sex/gender differences in auditory sensitivity and susceptibility to NIHL.

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APPENDIX IB

Evidence of a Common Pathway in Noise-Induced Hearing Loss and Carboplatin Ototoxicity*

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In spite of the differences in the nature of the insult, the hearing loss from ototoxic drugs and noise exposure share a number of similarities in cochlear pathology. This paper explores the common factors between noise-induced hearing loss and ototoxicity by experimentally manipulating cochlear glutathione (GSH). In the first experiment, chinchillas were treated with a drop of saline (50 µl) on the round window of one ear and a drop of buthionine sulfoximine (BSO, 50 µl of 200 mM) on the other ear. BSO is a drug that blocks GSH synthesis and it was hypothesised that GSH-depressed ears would be more vulnerable to noise. Six hours after treatment, the animals were exposed to a 105 dB 4 kHz octave band noise for 4 hours, then a second dose of BSO was applied 2 hours later. The BSO treated ears showed more temporary threshold shifts and reduced GSH staining at day 4 post exposure, but there was no BSO effect in terms of greater permanent threshold shift (PTS) or hair cell loss. In the second experiment, chinchillas were pretreated with BSO and 3 days later were given either a single dose of carboplatin (25 mg/kg i.p.), a double dose (day 3 and 7) or only BSO. Chinchillas that received BSO and the double dose of carboplatin had significantly greater loss of inner and outer hair cells than the carboplatin chinchillas. In addition, the BSO and carboplatin chinchillas also had larger decreases in evoked response amplitudes suggesting that GSH depletion potentiated the ototoxicity of carboplatin. These results are discussed in terms of the role of reactive oxygen species in creating hearing loss and the potential protective role of glutathione.

Keywords: noise-induced hearing loss, antioxidants, carboplatin, reactive oxygen species, glutathione, buthionine sulfoximine

Introduction

The hypothesis of a common pathway underlying the hearing loss caused by noise exposure and certain ototoxic drugs is based on similarities in their cochlear pathology and audiometric profiles. For example, noise exposures create a hearing loss that is related to the spectral characteristics of the traumatising exposure. For example, many industrial settings are associated with a relatively broad band noise that is enhanced by the resonance of the external auditory meatus to deliver a band of noise centred at approximately 3 kHz. Thus, the typical audiogram of noise induced hearing loss (NIHL) has either a peak at 4 kHz or has a loss at 4 and 8 kHz; aminoglycosides and platinum

compounds produce a hearing loss that starts at the highest frequencies. Both noise and drugs typically target the outer hair cells at the base of the cochlea with the stereocilia often being the first to show pathological changes such as "blebs" or fusion. With greater hearing losses the pathology becomes more pervasive including supporting cell damage, lesions in the stria vascularis and, in very severe cases, inner hair cell loss. As Hawkins (1973) pointed out almost 25 years ago, it is difficult to predict the cochlear pathology from knowing the pattern of the audiogram and, as a corollary it is difficult to identify the cause of the cochlear lesions from the pattern of cochlear pathology.

The similarity between the pathology associated with noise-induced hearing loss (NIHL) and drug-induced hearing loss may be a reflection of a common trigger, reactive oxygen species (ROS) leading to the sequela of cochlear pathology. Much of the data on the role of ROS in tissue damage comes from cell or organ culture experiments. Unfortunately, experiments with mammalian cochlear organ cultures have been limited to studies of ototoxic effects in the developing cochlea and there is no viable model for studying NIHL in cultures. Additional problems in studying the direct effects of ROS arise because of the short half life of ROS and the difficulty in accessing the living cochlea.

In spite of the lack of direct evidence, there are compelling reasons and strong circumstantial evidence for ROS and their potential role in hearing loss. Given the energy consuming operation of the cochlea, the generation of ROS is a normal part of homeostasis. However, with high levels of stimulation, the cellular respiratory process of the mitochondria cannot accommodate the higher levels of demand and produce excessive ROS. Thus, the balance between ROS and their normal reactions with cellular antioxidants shifts with an accumulation of ROS. In addition, high levels of stimulation lead to excitotoxic reactions and swelling of the afferent dendrites, as well as ischaemia as reflected in ROS accumulation at the stria vascularis. Yamane et al. (1995) showed the intimate correlation between strial blood flow and the presence of the superoxide radical $O_2^{\cdot -}$ in guinea pigs following noise exposure. They reported that immediately after a noise exposure strial blood flow was either greatly reduced or stopped and there was an accumulation of $O_2^{\cdot -}$ along the endolymph margin of the stria. Two hours later, blood flow had resumed and the $O_2^{\cdot -}$ concentration was greatly reduced; six hours later blood flow was normal and $O_2^{\cdot -}$ was not detectable. The implication of the Yamane et al. (1995) results is that noise exposure creates cochlear ischaemia with an increase in $O_2^{\cdot -}$ presence. The toxic $O_2^{\cdot -}$ and related ROS are then responsible for assaults on the cell membrane system, structural proteins and cell nucleus.

Additional evidence that ROS contribute to NIHL comes from pharmacological studies of prevention with R-phenylisopropyladenosine (R-PIA). Studies by Hu et al. (1997) and Liu et al. (1999) showed that the hearing loss and hair cell damage from exposure to either a 105 dB SPL 4 kHz octave band (OB) noise or a 150 dB peak SPL impulse noise could be reduced with a prior application of R-PIA to the round window. These results are exciting in terms of their clinical implications but they are ambiguous in terms of the cellular mechanisms involved. R-PIA is a selective adenosine receptor agonist that has been found to upregulate the activity of several antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, and catalase (Maggirwar et al., 1994). R-PIA is also a potential inhibitor of glutamate synthesis and a stimulus for nitrous oxide (NO) production. All three of these effects of R-PIA have positive

implications for reducing hearing loss. For example, R-PIA may reduce ROS-mediated damage by stimulating antioxidant production; blocking glutamate might reduce cytotoxicity seen at afferent auditory nerve dendrites following noise (Spoendlin, 1976); or increased NO availability might stimulate blood flow to the cochlea which would partially reduce ischaemic conditions.

The source for an increase in ROS may be quite different for drugs such as cisplatin and carboplatin. Cisplatin and carboplatin are members of a family of platinum-based drugs that are used in the treatment of various types of tumours in humans. One potential side effect of the anti-cancer drugs is hearing loss, particularly at high frequencies (Frelich et al., 1996; McKeage, 1995; Macdonald et al., 1994). Platinum cytotoxicity has been shown to be accelerated or enhanced in the presence of ROS, and decreased by antioxidants, particularly glutathione (GSH), α thiol/sulfhydryl compound consisting of γ-glutamate, γ-cysteine and glycine (Ahn et al., 1994; Arrick and Nathan, 1984; Babu et al., 1995; de Graeff et al., 1988; Eastman, 1987; Ferguson, 1995; Lai et al., 1989; McGinness et al., 1978; Mistry et al., 1993; Russo et al., 1986; Tonetti et al., 1993; Walker and Gale, 1981). Clear correlations have been found between GSH levels and sensitivity to platinum compounds (Ahn, 1994; Arrick and Nathan, 1984; Meijer et al., 1990, 1992; Mellish et al., 1993; Mistry et al., 1991). Cells with inherent or acquired resistance to platinum compounds have higher levels of GSH than susceptible cells, and depletion of cellular GSH can increase sensitivity to damage. In organ culture, cisplatin has been shown to increase superoxide dismutase (SOD), catalase and malondialdehyde activities, while reducing the activity levels of GSH, GSH-peroxidase and GSH-reductase (Ravi et al., 1995). In vitro experiments are interesting, but it can be risky to simply extrapolate the results to the organ system (ear) because the state of equilibrium between ROS and the set of antioxidant molecules is complex. Consequently, when the ear is poisoned with cisplatin, the normal antioxidant defence system is disrupted and may be unable to keep up with the normal ROS that are generated when the ear is stimulated. By contrast, high levels of noise exposure produce an increase in ROS (Yamane et al., 1995a,b).

The common pathway hypothesis may help explain the particularly traumatic interaction seen with cisplatin and noise. Gratton et al. (1990) reported that cisplatin and noise exposures that were individually non-traumatic could combine to produce substantial hearing loss and hair cell damage in chinchillas. If cisplatin depletes GSH and other components of the antioxidant defence system (Ravi et al., 1995), then the ear would be expected to be even more vulnerable to the ROS generated by the noise exposure. The common pathway with noise and certain ototoxic drugs which leads to cochlear pathology appears to be toxic ROS. However, the processes leading to an accumulation of ROS may be quite different.

The following two experiments continue to explore the commonality between the patho-physiology of noise- and drug-induced hearing loss by explicitly blocking one component of the antioxidant pathway, GSH, and observing the effects on hearing loss and hair cell loss in chinchillas. In both experiments, buthionine-sulfoximine (BSO) was used to inhibit g-glutamyl cysteine synthtase (GCS) which is the rate limiting enzymes for GSH synthesis. In Experiment I, a small dose of BSO was applied to the round window membrane before and after animals were exposed to high-level noise.

In Experiment II, BSO was chronically infused into the cochlea via osmotic pumps before and after animals were treated with carboplatin. Carboplatin is related to cisplatin and is used in

treatment with a number of cancers because it has less severe side effects than cisplatin. In people, guinea pigs and rats, carboplatin produces a high-frequency loss with a basal lesion of OHC. Carboplain in the chinchilla produces a loss of IHC with virtually no loss of OHC as decrease in distortion product. The introduction of BSO with carboplatin will provide a perspective on thc importance of glutathione with the unique IHC loss of the chinchilla. The results support the notion of a common pathway leading to NIHL and carboplatin ototoxicity.

Experiment I: BSO alters thc course of recovery from NIHL

BSO is a specific inhibitor of g-glutamylcysteine synthetase (GCS), the rate-limiting enzyme in the synthesis of GSH from its constituent amino acids (Gao et al., 1994; Grieshaber, 1989; Griffith and Meister, 1979; Hoffman et al., 1988; Meister, 1991, 1995). Because BSO treatment blocks GSH synthesis and leads to reduced levels of GSH both in vitro and in vivo, it has been used as a tool for depleting GSH in a variety of cells and tissues, including the cochlea (Lazenby et al., 1988; Murray et al., 1986). Given the role of GSH in the cellular antioxidant system, the fact that GSH is upregulated with noise exposure (Bobbin et al., 1995), and the facilitative effects of R-PIA on recovery from noise-induced damage (Hu et al., 1997), we hypothesised that BSO-treated ears would have larger noise-induced threshold shifts and greater hair cell (HC) losses than saline-treated control ears.

Methods

Sixteen adult chinchillas served as subjects. Animals were divided into noise-exposed (N=12) and non-exposed (N=4) groups. Two noise-exposed animals were sacrificed 4 days after exposure for mercury orange staining to look at GSH distribution in the hair cells, while the other 10 noise-exposed animals were sacrificed along with non-exposed animals at 20 days post-exposure for hair cell counts. Procedures for the care and use of all animals in our studies were approved by the University of Buffalo Institutional Animal Care and Use Committee.

Each animal was anaesthetised with an intramuscular injection of ketamine (60 mg/kg) and acepromazine (0.5 mg/kg), and recording electrodes were implanted in each inferior colliculus (IC) and in the rostral cranium. After two or more weeks of recovery, the animals were again anaesthetised, and small holes were made in the bullae. Approximately 50 µl of 200 mM BSO (Sigma Chemicals) in physiological saline was dropped onto the right round window, and an equal volume of physiological saline was dropped onto the left round window. Stainless steel tubes were implanted above the round window niches for application of a second dose after the noise exposure. The tubes were sealed to the bulla with dental cement, and the skin incision was sutured. We applied the first dose of BSO at 6 h before noise exposure and a second dose via the stainless steel tubes at 2 h after noise exposure. The purpose of the two-dose schedule was to maximise the likelihood of depleting GSH during the early stage of hair cell recovery. After the second BSO application, the tubes were removed and the holes in the bullae were sealed with dental cement.

Animals in the noise-exposed group were exposed to a 4 kHz OB noise at 105 dB SPL (re: 20 mPa) for 4 h. The noise was generated by a D/A converter on a digital signal processing board (DSP) in a personal computer (PC), and routed through a manual attenuator (HP 350D), a filter, and a low-distortion power amplifier (NAD 2200) to an acoustic horn (JBL 2360) suspended directly above the cages in a sound booth. Noise levels were measured using a Type I sound level meter (Larson and Davis 800B) and a ½" microphone (Larson and Davis, LDL

2559), with the microphone positioned within the cage at the level of an animal's head.

The effects of GSH depletion on cochlear function were assessed by recording auditory evoked potentials from the IC (IC-EVPs) and cubic distortion product otoacoustic emissions (DPOAEs). For IC-EVP testing, stimuli were digitally generated tones (10 ms duration, 5 ms cosine rise/fall) at 1, 2, 4, 6, and 8 kHz, presented at a rate of 10/sec. The stimuli were routed through a computer-controlled attenuator to an insert earphone (Etymotic Research ER-2). The output of the insert earphone was calibrated before each test. Electrical activity from the recording electrode was amplified (20,000 X) and filtered (10-3000 Hz) by a Grass P511K bioamplifier, and directed to an A/D converter on a DSP board in the PC. Stimulus level was varied in 5 dB steps from 0 to 80 dB SPL. One hundred samples were averaged at each level. Threshold was defined as the mid-point between the lowest level at which a clear response was seen and the next lower level where no response could be discerned. The thresholds of IC-EVPs were determined before BSO application and at various times after the noise exposure (15 minutes; 1, 2, 4 and 20 days).

For DPOAE testing, pure tone stimuli (f_1 and f_2) were generated by two DSP boards in a PC, and sound levels in the ear canal were measured using a low-noise probe microphone. Input/output (I/O) functions were recorded in 5 dB steps from 0 to 80 dB SPL, using equal-level primaries and an f_2/f_1 ratio of 1.2. I/O functions were collected at f_1=2, 4, and 8 kHz, in random order. The animals were held in a custom-built restraining device (Snyder and Salvi, 1994) and tested while awake. At least two sets of I/O functions were obtained prior to the noise exposure, and the average of these measures served as the baseline. DPOAEs were also measured at 15 minutes, and 1, 2, 4, and 20 days after noise exposure.

All noise-induced threshold shifts (TSs) were calculated relative to the threshold measured before the application of BSO or saline. The mean TSs of the BSO- and saline-treated ears were compared using Student t-tests. For comparison of DPOAE amplitudes, mean amplitudes for stimuli at 60 dB SPL (L_1=L_2) were compared as a function of treatment (BSO versus saline) with Student t-tests.

After final auditory tests had been completed, the animals were anaesthetised with a lethal dose of sodium pentobarbital and decapitated. Each bulla was quickly removed from the skull, and the cochleas were slowly perfused through the round window with a succinate dehydrogenase (SDH) staining solution. The apex of the cochlea was removed, and the cochlea was immersed in the SDH staining solution for 1 h at 37°C, followed by immersion in 10% formaldehyde for 24 h. The cochlea was dissected and sections of the organ of Corti were mounted in glycerin on glass microscope slides and coverslipped. The specimens were examined for HC losses under a light microscope.

We also examined GSH staining in two animals sacrificed 4 days after noise exposure. Both left and right bullae were quickly removed and the cochleas were exposed. The ossicles were removed, the oval window was opened, and 50 mM mercury orange (1-4-chloro-mercury-phenyl-azo-2-naphthol; Sigma Chemicals) in toluene was gently perfused through the round window membrane with a fine pipette. The cochleas were then immersed in the same solution for 20 minutes at room temperature, rinsed in two changes of toluene, and fixed with 10% formalin in phosphate buffer (pH 7.4) for 1 hour. The cochleas were dissected in phosphate buffer and the organ of Corti sections were

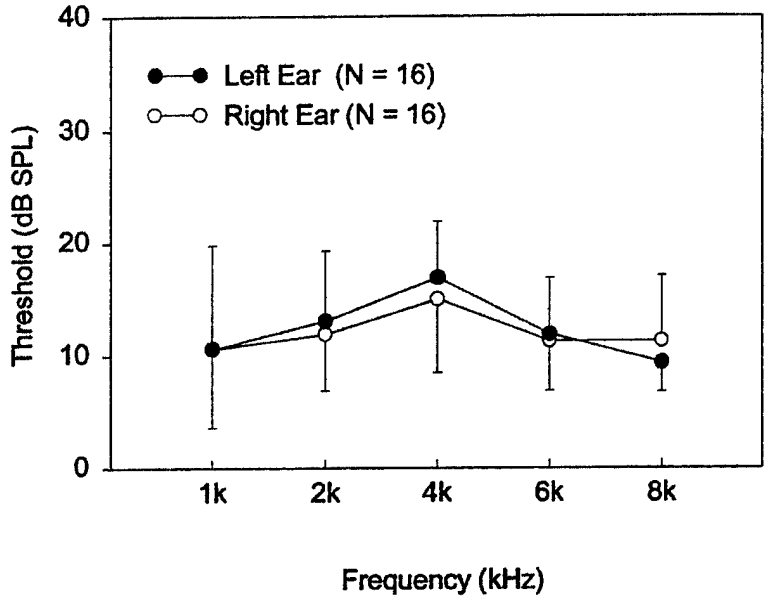

Figure 1. Average IC-EVP potential thresholds for left and right ears of 16 chinchillas prior to application of BSO or saline. Bars show standard deviations.

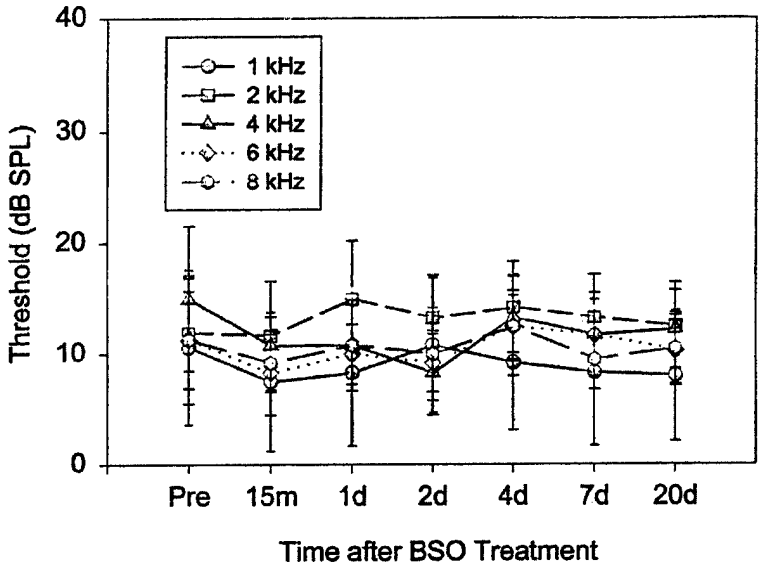

Figure 2. IC-EVP thresholds of animals in the non-exposed (BSO only) group, measured at various times after BSO application.

mounted in 50% glycol on glass microscope slides for examination with a Bio-Rad MRC 1000 laser scanning confocal unit attached to a Nikon microscope. Optical sections were collected at 0.5 mm intervals through the organ of Corti.

Results

The mean IC-EVP thresholds for right ears (open circles) and left ears (filled circles) of all 16 chinchillas before the BSO and saline treatment are shown in Figure 1. There were no significant differences between right and left ears at any frequency prior to drug application. Figure 2 shows IC-EVP thresholds as a function of time and frequency for the four non-exposed animals.

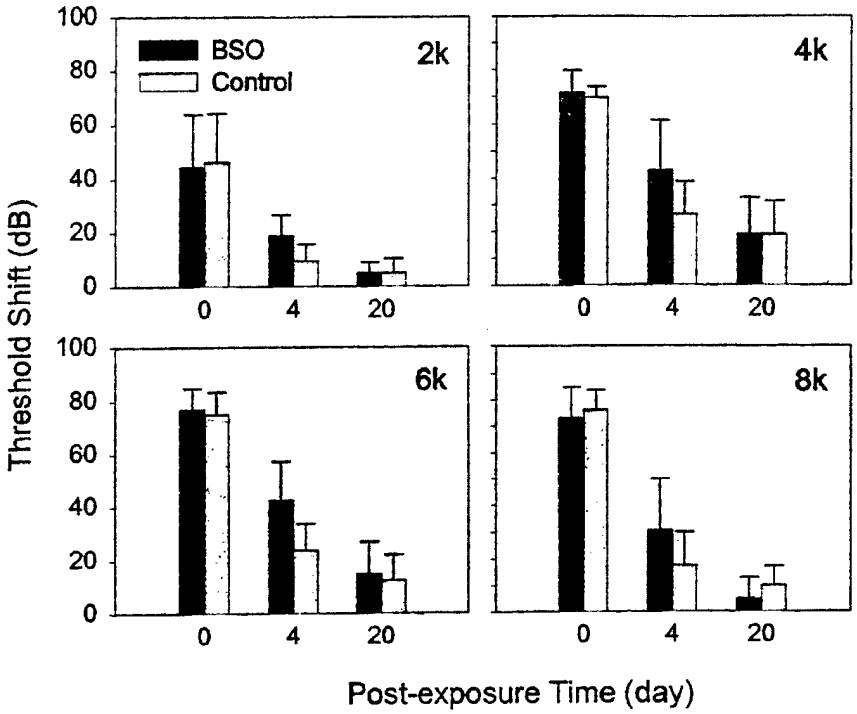

Figure 3. Average evoked potential threshold shifts for BSO-treated right ears and saline-treated left ears after exposure to a 105 dB SPL 4 kHz OB noise for 4 h. Twelve animals were exposed to the noise; two of these were sacrificed after testing on day 4. Threshold shifts were significantly different between BSO and control ears at 4, 6 and 8 kHz, four days after noise exposure.

IC-EVP thresholds remained stable over the 20-day period, indicating that the BSO treatment alone had no effect on auditory sensitivity.

Figure 3 compares mean TSs of the BSO treated ears and the control ears 15 minutes (0 days; n=12 animals), 4 days (n=12 animals), and 20 days (n=10 animals) after noise exposure at 2, 4, 6 and 8 kHz. The mean TS measured 15 min after the noise exposure was 66.6 dB. There were no significant differences in TSs at this point between BSO-treated and control ears. One to two days after noise exposure, thresholds of the BSO-treated ears had recovered 4.4 to 12.7 dB less than thresholds of the saline-treated ears at 4 and 8 kHz. However, these differences were not statistically significant. Four days after noise exposure, the BSO-treated ears showed 9.4, 16.3, 18.8, and 13.1 dB less recovery at 2, 4, 6 and 8 kHz, respectively. Differences between left and right ears were significant at 4, 6 and 8 kHz (p = 0.018, 0.002 and 0.044, respectively). Although BSO-treated ears showed less recovery between 0 and 4 days after exposure, there were no significant differences between ears 20 days after noise exposure.

Figure 4 shows DPOAE amplitudes for 2, 4 and 8 kHz stimuli measured in saline-treated ears (solid lines) and BSO-treated ears (dashed lines). The solid lines without symbols show DPOAEs measured prior to drug treatment and noise exposure. Lines with symbols show DPOAE amplitudes measured at 15 minutes (top panel), 4 days (middle panel) and 20 days (bottom panel) following the noise exposure. Amplitudes at all three frequencies decreased significantly 15 minutes after exposure, but the magnitude of loss was not significantly different between the BSO-treated ears and the saline-treated ears. DPOAE

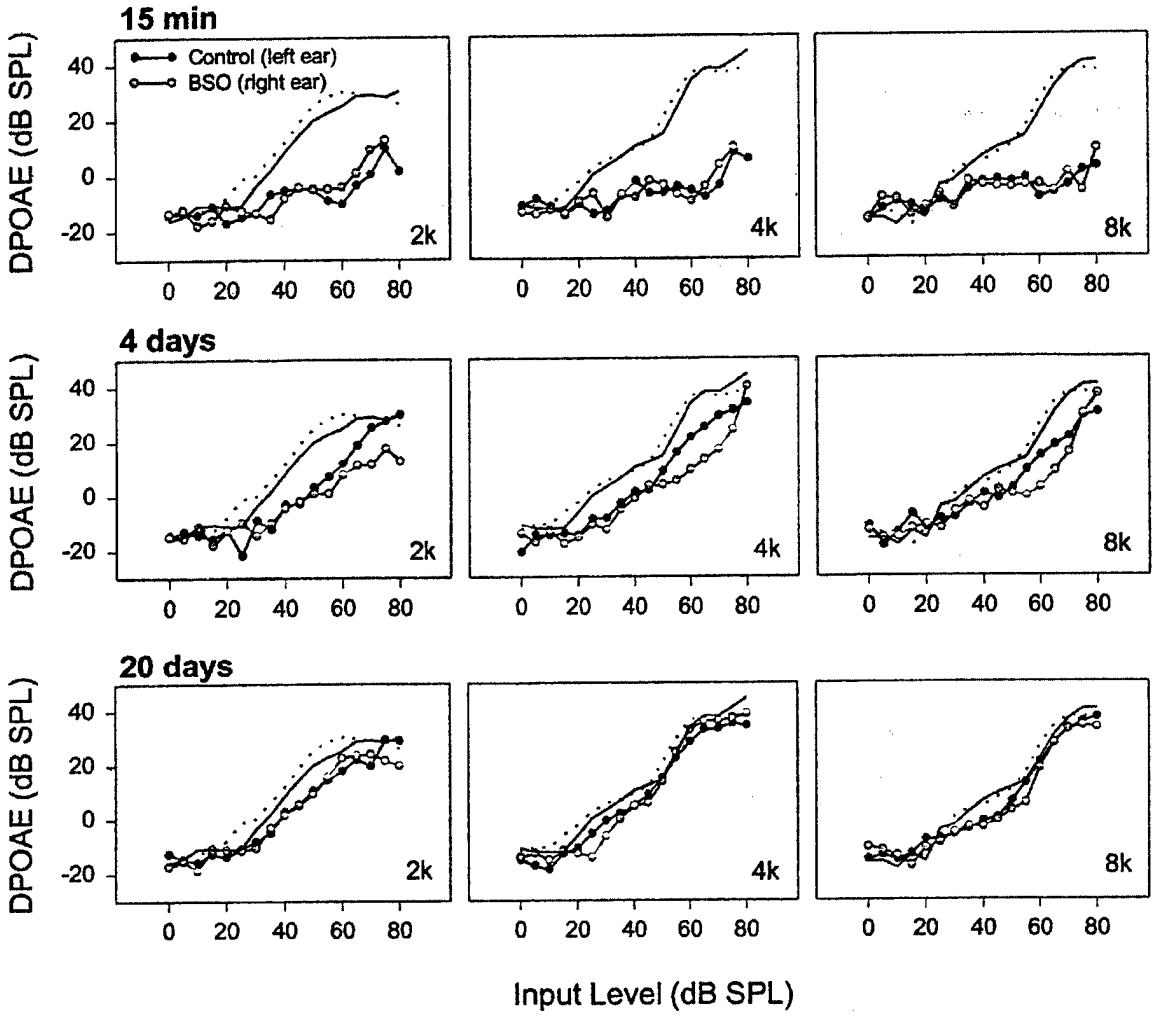

Figure 4. DPOAE input/output functions before and after (15 min, 4 days, 20 days) exposure to the noise. Solid and dashed lines without symbols represent pre-exposure values for left (n=12) and right (n=12) ears, respectively. Filled circles are for saline-treated ears; open circles are for BSO-treated ears. Significant differences between BSO and control ears were observed at 2, 4 and 8 kHz, four days after noise exposure.

amplitudes recovered substantially between 15 minutes and 4 days, with greater recovery in saline-treated ears than in BSO-treated ears. On Day 4, BSO-treated ears showed 12.0 and 11.5 dB less recovery at 60 dB SPL at 4 and 8 kHz ($p = 0.012$ and 0.024, respectively). By 20 days after noise exposure, however, the DPOAE amplitudes of both experimental and control ears had essentially recovered to the pre-exposure values and differences between BSO- and saline-treated ears were negligible.

Two noise-exposed animals were sacrificed 4 days after exposure for mercury orange staining. The IHC were only weakly stained in all four ears, and no differences were seen between the BSO-treated ears and the control ears. In contrast, there were obvious and consistent differences in staining patterns of OHCs. Figure 5 illustrates mercury orange staining in the region of the cochlea with the largest OHC lesions, at an optical level of 6-8 μm below the cuticular plate. The upper panel shows mercury

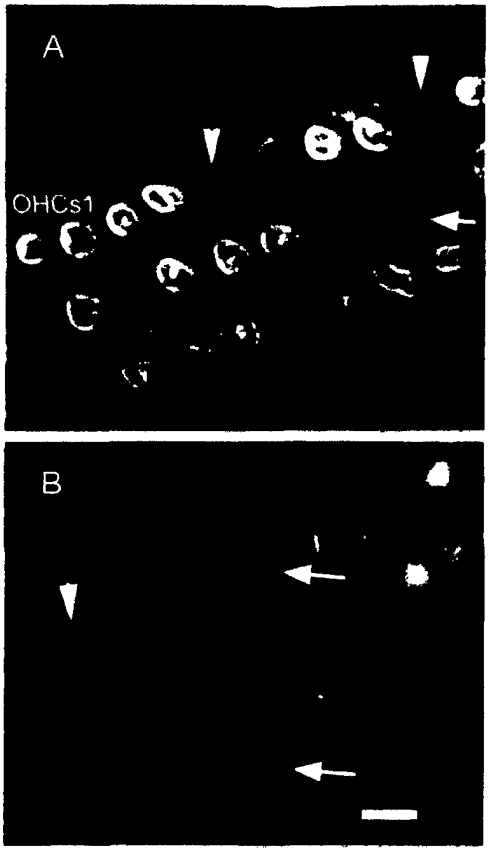

Figure 5. Mercury orange staining of saline-treated (A) and BSO-treated (B) organ of Corti four days after exposure to noise, in the region of most severe cochlear damage. Note the greater degree of edema (arrows) and reduced staining in BSO-treated ear.

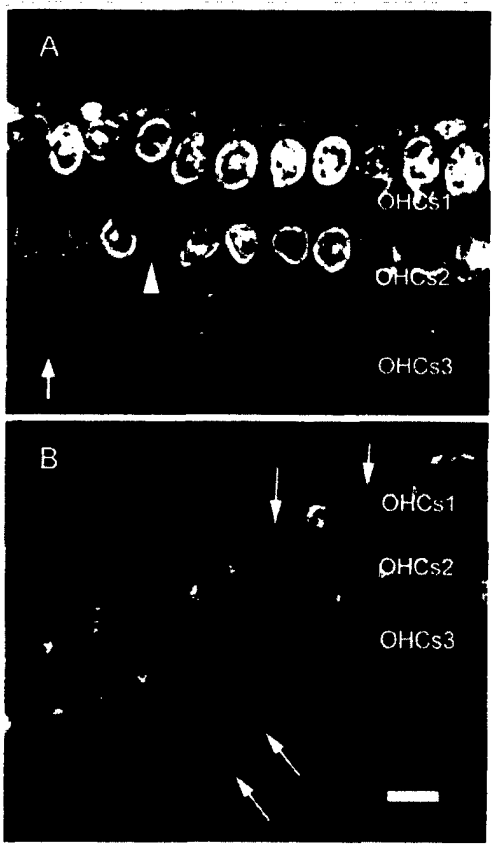

Figure 6. Same cochleas as shown in figure 5, but in a region apical to the location of maximum damage. The saline-treated ear (A) has much stronger staining in OHC rows 1 and 2, and less cell edema and distortion of cell bodies.

orange staining in a saline-treated ear. Note that areas where OHCs are missing (arrow heads) are completely devoid of staining, whereas most surviving OHCs show a normal GSH staining pattern, characterised by a ring-like structure with strong central staining. Only a few OHCs showed enlarged cell bodies with weakly-stained central portions (arrows). The BSO-treated ears exhibited GSH staining patterns (lower panel) that were much different from controls. Enlarged OHCs, which usually lacked the strong bolus of central staining (arrows), appeared to be more numerous. Normal-sized OHCs also showed different staining patterns. The normal ring-like structure was replaced by relatively even mercury orange staining throughout the cytoplasm.

Figure 6 shows mercury orange staining in an area of the cochlea where relatively little noise-induced HC loss occurred. The upper panel shows staining in a saline-treated ear and the lower panel shows staining in a BSO-treated ear. Note that enlarged OHCs are more numerous in the BSO-treated ear than in the saline-treated ear. Despite differences between BSO- and saline-

treated ears in GSH staining at 4 days post-exposure, there were no differences in HC loss at 20 days post-exposure.

Discussion

The dose of BSO used in this study did not interfere with normal function or survival of cochlear HCs. However, application of BSO to the round window membrane altered the pattern of recovery of IC-EVP thresholds and DPOAE amplitudes following noise exposure. There were no differences between the BSO- and saline-treated ears immediately after the exposure, suggesting that GSH depletion did not potentiate the initial noise-induced damage to the hair cells. However, 4 days after noise exposure, physiologic measurements showed significantly less recovery in BSO-treated ears. Consistent with this, mercury orange staining at 4 days post-exposure showed reduced GSH levels in BSO-treated ears.

The reason why the effects of BSO were evident 4 days after noise exposure and not earlier or later is not clear. The simplest explanations are related to the dose and time course of BSO application. It seems likely that the small dose of BSO used in this experiment produced only modest decreases in GSH levels, and that GSH levels were able to recover relatively rapidly, before permanent damage could occur. Another possibility to consider is that the acute application of BSO may have caused a compensatory increase of other antioxidant enzymes. If this is the case, then more general suppression of the antioxidant system should result in greater permanent deficits than we observed here.

In the saline-treated ears, areas of missing OHCs were devoid of staining. The surviving OHCs generally showed normal shapes and a normal staining pattern, characterised by staining concentrated in the central portion and around the perimeter of the cells. In contrast, the BSO-treated ears showed variable staining patterns. Some OHCs had enlarged cell bodies, suggesting that swelling had occurred. Others, even those of apparently normal size, lost the normal pattern of staining, and mercury orange staining became diffusely distributed throughout the cytoplasm. In addition, in less damaged areas of the cochlea, the number of swollen OHCs appeared to be greater in BSO-treated ears than in saline-treated ears.

One limitation of the mercury orange staining method is a lack of specificity, because it reacts rapidly with other low molecular weight thiols such as cysteine (Murray et al., 1986). Therefore, the residual staining seen in BSO-treated ears cannot be attributed specifically to GSH. However, because BSO is a specific inhibitor of GSH synthesis, it is reasonable to attribute any differences in staining patterns to BSO-induced depletion of GSH. While staining was not eliminated in BSO-treated ears, its distribution was altered dramatically, and the alteration was correlated with an altered pattern of recovery after noise exposure.

Summary and Conclusions

The results of Experiment I suggest that ears with decreased levels of GSH are more vulnerable to noise trauma than normal ears of the same animals. Increased vulnerability was evident in two separate physiological measures, IC-EVPs and DPOAEs, and correlated with diminished mercury orange staining of GSH and other cellular thiols. The effects were modest and temporary, appearing within 4 days after exposure and dissipating by 20 days, probably as a consequence of our BSO dosing parameters. It is likely that more profound and permanent effects would have been seen with chronic administration of BSO throughout the recovery period. In the next experiment, we used osmotic pumps to infuse BSO directly into the cochlea

for an extended period of time, in order to test the hypothesis that GSH depletion potentiates HC damage from carboplatin.

Experiment II: GSH Depletion Potentiates Carboplatin Ototoxicity

The ototoxic effects of carboplatin have been studied extensively in guinea pigs and chinchillas. In guinea pigs, the pattern of HC loss caused by carboplatin represents the typical response of the mammalian cochlea to ototoxic drugs and noise, i.e., greater vulnerability of OHCs versus IHCs, and greater vulnerability of basal HCs versus apical HCs (Schweitzer et al., 1986; Takeno et al., 1994a,b). Consequently, guinea pigs treated with carboplatin develop a hearing loss that is primarily high-frequency in nature (Taudy et al., 1992). The chinchilla, by contrast, has an atypical response to carboplatin (but not to other ototoxic drugs or noise). For reasons that are not yet known, carboplatin selectively destroys IHCs throughout the cochlea of the chinchilla, leaving OHCs intact except at high doses (Hofstetter et al., 1997a,b; Takeno et al., 1994a,b; Wake et al., 1993, 1994). The ability to selectively destroy IHCs while leaving OHCs intact provides us with unprecedented opportunities to study the effects of IHC loss independent of OHC loss. Studies using the unique carboplatin-treated chinchilla model of IHC loss have confirmed that the IHCs make little or no contribution to DPOAEs (Hofstetter et al., 1997a; Jock et al., 1996; Trautwein et al., 1996), and have led to the unexpected finding that thresholds of cochlear potentials and IC-EVPs remain normal with moderate-to-large losses of IHCs as long as OHCs remain intact (Burkard et al., 1997; Jock et al., 1996; McFadden et al., 1998; Trautwein et al., 1996).

Previous studies have implicated GSH and GSH-dependent enzymes in platinum toxicity. With respect to ototoxicity, Ryback et al. (1995) showed that administration of diethyldithiocarbamate, a compound that increases GSH and GSH-peroxidase activity, significantly reduced threshold shifts in rats treated with cisplatin, and Ravi et al. (1995) demonstrated that cochlear GSH is down-regulated with application of cisplatin. The purpose of the present study was to determine if carboplatin ototoxicity in the chinchilla cochlea is enhanced when GSH synthesis is inhibited. The severity of carboplatin ototoxicity was assessed using the same physiological measures and procedures used in Experiment I, and these measurements were complemented by cochleograms showing IHC and OHC losses. Since previous studies have shown that carboplatin preferentially damages the IHCs in chinchilla, we were particularly interested to see if GSH depletion would increase the amount of damage to the OHCs.

Methods

Twelve chinchillas were randomly divided into three groups of 4 animals each: a single-dose carboplatin group that received BSO in the right ear, followed 3 days later by a single dose of carboplatin (25 mg/kg i.p.); a double-dose carboplatin group that received BSO in the right ear followed by two doses of carboplatin (25 mg/kg i.p. X 2), at 3 and 7 days after the beginning of BSO treatment; and a *drug control group*, that only received BSO in the right ear without any carboplatin.

Each subject was anaesthetised with a mixture of ketamine (60 mg/kg) and acepromazine (0.5 mg/kg), and tungsten recording electrodes were stereotaxically implanted into the left and right IC as described for Experiment I. Osmotic pumps (2ML4, Alza Corporation) (Brown et al., 1993; Schindler et al., 1995) were also implanted in each animal. The right bulla was exposed through a posterior approach and a small hole was drilled in the bony wall of the basal turn of the cochlea. A stainless steel tube was inserted into scala tympani and sealed to the cochlea with silicone glue. Polyethylene tubing connected the stainless steel tube to the osmotic pump. The

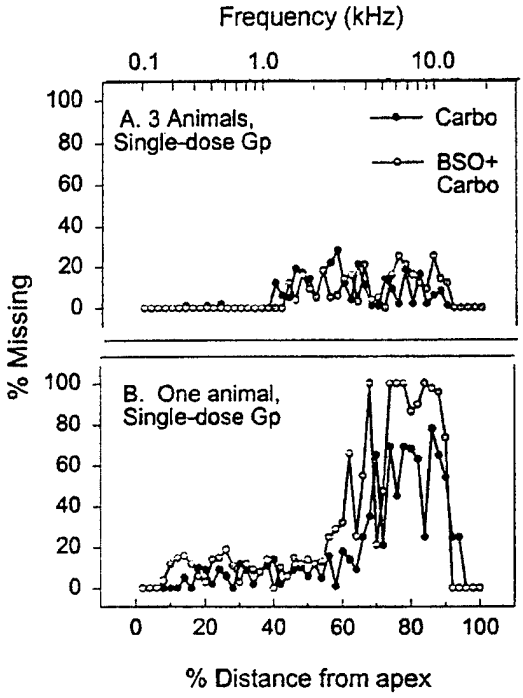

Figure 7. Cochleograms showing IHC losses of animals in the single-dose carboplatin group. A. Average IHC loss of three animals with minor lesions. B. IHC loss of the one subject in the single-dose group with a relatively severe IHC loss. There was no appreciable loss of OHCs in any of the ears.

pump was implanted under the skin on the back of the neck, and the incision was sutured closed. The pump, pre-filled with 15 mM BSO in Hanks balanced salt solution, had an infusion rate of 5 µl/hr. The infusion period lasted 14 days.

Three days after implanting the osmotic pump, animals in the two carboplatin groups were injected with carboplatin. Animals in the double-dose group received a second dose of carboplatin 4 days after the first.

Results

Thresholds and I/O functions of IC-EVPs and DPOAEs were examined before and at various times during the BSO infusion. Drug control subjects showed small losses of sensitivity (5-10 dB on average for IC-EVPs) at 2 days after beginning the BSO treatment, but complete recovery of thresholds and response amplitudes by 7-14 days. When animals in the drug control group were sacrificed for histology after 14 days of BSO infusion, no IHC or OHC losses were observed. Thus, both physiological and anatomical measures show that GSH depletion causes no direct cochlear damage.

Three animals in the single-dose carboplatin group showed relatively minor IHC losses from carboplatin, and no significant threshold shifts. As shown in Figure 7A, the average IHC loss of these animals was only 10-30% in the 1-10 kHz region of the cochlea, and there were no significant differences between treated and non-treated ears. In contrast, one animal in the single-dose group showed large IHC losses (Figure 7B), and a significant difference ($p < 0.001$) between the BSO-treated ear and the normal ear. The average IHC loss for this animal was 18.74% ± 4.6 in the control ear, versus 29.97% ± 2.74 in the BSO-treated ear. OHC loss was negligible in all ears.

The most pronounced effects of GSH depletion were seen in the double-dose carboplatin group. Both BSO-treated and control ears showed a significant decrease in IC-EVP response amplitudes after carboplatin treatment, with

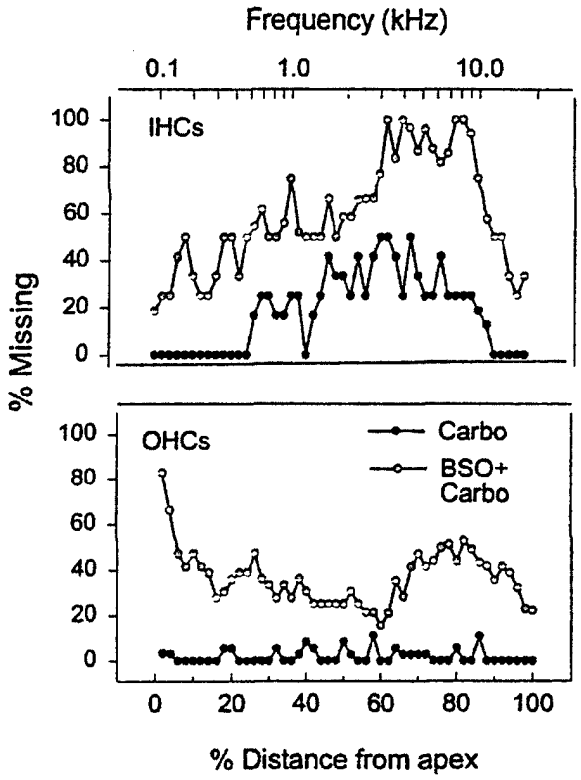

Figure 8. Average HC losses for animals in the double-dose group. Differences between BSO-treated ears (open circles) and control ears (filled circles) were significant for both IHC losses (top panel) and OHC losses (bottom panel)

slightly greater decreases in BSO-treated ears. Interestingly, control ears showed no significant change in DPOAE amplitude after carboplatin treatment, while BSO-treated ears exhibited decreased amplitudes at 2, 4, and 8 kHz. At primary levels of 60 dB SPL, the BSO-treated ears had amplitude shifts of 4-12.3 dB relative to control ears and pre-drug values.

The most dramatic differences between ears were seen in HC losses. As shown in Figure 8, the double dose of carboplatin not only produced more IHC loss in BSO-treated ears (top panel), but also significant OHC loss (bottom panel). Carboplatin produced an average IHC loss of 18.1% ± 4.62 in control ears versus 59.1% ± 2.71 in BSO-pretreated ears. Control ears showed scattered OHC losses along the organ of Corti, with an average OHC loss of only 1.9% ± 8.8. In contrast, the average OHC loss for the BSO-treated ears was 36.7% ± 6.0. Both IHC and OHC differences were statistically significant ($p < 0.001$).

Discussion

The results show that infusion of BSO (15 mM) directly into the chinchilla cochlea potentiates the ototoxicity of carboplatin. Potentiation was seen in one of four animals treated with a single low dose of carboplatin, and in all four animals in the double-dose group. The differences between the BSO-treated ears and the control ears of animals in the double-dose carboplatin group were dramatic. BSO-treated ears showed approximately 41% more IHC loss than control ears, and significantly more OHC loss (37%, versus 2%) as well.

The results are consistent with the idea that carboplatin treatment itself reduces cellular GSH, as has been shown to occur following cisplatin treatment (Ravi et al., 1995) and that

the combined effects of BSO and carboplatin depleted GSH levels beyond a critical level for producing permanent damage. Presumably, permanent damage occurs only when the scavenging capacity of the antioxidant defence system falls below a critical level relative to ROS production. The hypersensitivity of the one chinchilla in the single-dose carboplatin group could be an indication of a generally less efficient antioxidant defence system or a heightened production of ROS in this animal. In future studies, it will be useful to develop techniques for assaying cochlear levels of GSH and other antioxidant enzymes and correlating them with individual susceptibility to address these issues directly.

The most intriguing finding of this study was the potentiation of OHC loss in BSO-treated ears, since previous studies have clearly established that OHCs in the chinchilla are much less susceptible to carboplatin than IHCs. In the current study, OHC loss was minor in all control ears and BSO-treated ears of animals in the single-dose group. Consistent with this, DPOAEs, which rely on OHCs for their generation (Trautwein et al., 1996), were normal in these ears. In contrast, BSO-treated ears of animals in the double-dose group had OHC losses scattered throughout the cochlea, resulting in reduced DPOAE amplitudes. One interpretation of these results is that OHCs in the chinchilla cochlea are susceptible to carboplatin only when antioxidant enzyme levels fall below a critical level.

Previous studies have shown that the sensitivity of tumour cells to platinum drugs is related to intracellular levels of GSH and related enzymes (e.g., Meijer et al., 1992; Mellish et al., 1993; Mistry et al., 1991). Inherent and acquired resistance to platinum drugs through up-regulation of intracellular GSH poses an important problem for cancer treatment. Although studies show that decreasing cellular GSH levels by pre-treatment with BSO can increase the platinum sensitivity of some resistant tumour cell lines (Lee et al., 1992; Meijer et al., 1992; Mistry et al., 1991; Singh et al., 1995), our results suggest that this approach may potentiate ototoxic damage. Developing techniques for increasing the sensitivity of tumour cells to chemotherapy while avoiding increased damage to cochlear hair cells is an important challenge for both basic and clinical research.

Summary and Conclusions

Since BSO is a specific and irreversible inhibitor of g-GCS, the effects of BSO on susceptibility to both noise and carboplatin are most likely mediated by inhibition of cochlear GSH synthesis (Kera et al., 1989; Kisera et al., 1995). The results of the two experiments described above provide strong circumstantial evidence of a common pathway for damage from noise and carboplatin.

Acknowledgements

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APPENDIX IC

ELSEVIER

Hearing Research 128 (1999) 125–134

HEARING RESEARCH

Intracochlear infusion of buthionine sulfoximine potentiates carboplatin ototoxicity in the chinchilla

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Abstract

The aim of this experiment was to determine if buthionine sulfoximine (BSO), an inhibitor of glutathione (GSH) synthesis, enhances the ototoxicity of carboplatin. Osmotic pumps were used to infuse BSO into the right cochleas of 12 adult chinchillas for 14 days. The left cochleas served as controls. Animals were assigned to three groups: a drug control group that did not receive carboplatin, a group that received a single dose of carboplatin (25 mg/kg i.p.), and a group that received a double dose of carboplatin (25 mg/kg i.p. $\times 2$), with 4 days between injections. Carboplatin was administered after three days of BSO pre-treatment. Ototoxicity was assessed with evoked potentials recorded from electrodes implanted in the inferior colliculi (ICPs), distortion product otoacoustic emissions (DPOAEs), and cochleograms. BSO infusion itself caused no long-term functional or morphological changes. One of four animals treated with a single dose of carboplatin showed a significant loss of inner hair cells (IHCs), with greater loss in the BSO-treated ear. All animals in the double-dose carboplatin group showed marked differences between BSO-treated and control ears. Average IHC losses were 59% in BSO-treated ears vs. 18% in control ears. Moreover, BSO-treated ears sustained significantly greater outer hair cell (OHC) losses than control ears (37% vs. 2%, respectively). ICP and DPOAE response amplitudes were reduced slightly in BSO-treated ears relative to control ears, consistent with their greater hair cell loss. The results clearly show that BSO can enhance carboplatin ototoxicity in the chinchilla, supporting a role of GSH and reactive oxygen species in platinum ototoxicity.

Key words: Antioxidant; Chemotherapeutic drug; Cochlea; Hearing loss; Hair cell; Platinum

1. Introduction

Carboplatin is one of several platinum-based anticancer drugs that have been used to treat various types of solid tumors in humans (Bonomi, 1991; Canetta et al., 1985; Ruckdeschel, 1994; Weiss and Christian, 1993). For many patients being treated with platinum compounds, an unfortunate side effect is permanent hearing loss, particularly at high frequencies (Freilich et al., 1996; McKeage, 1995; Macdonald et al., 1994). Understanding the factors that make the cochlea vulnerable to damage from carboplatin and other platinum compounds may lead to ways of protecting patients from ototoxic damage.

The effects of carboplatin on cochlear hair cells (HCs) and hearing have been studied extensively in two animal models, the guinea pig and the chinchilla. In carboplatin-treated guinea pigs, the pattern of HC loss represents the typical response of the mammalian cochlea to ototoxic drugs and noise, i.e., greater vulnerability of outer hair cells (OHCs) vs. inner hair cells (IHCs), and greater vulnerability of basal (high-frequency) HCs vs. apical (low-frequency) HCs (Schweitzer et al., 1986; Takeno et al., 1994a,b). Consequently, carboplatin treatment results in significant high-frequency hearing loss in the guinea pig (Taudy et al., 1992). In contrast, chinchillas treated with carboplatin

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develop a very unusual pattern of HC loss. For reasons that are not yet known, IHCs of the chinchilla are much more susceptible to carboplatin than OHCs. Carboplatin selectively destroys IHCs throughout the cochlea of the chinchilla, leaving OHCs intact except at high doses (Hofstetter et al., 1997a,b; Takeno et al., 1994a,b; Wake et al., 1993, 1994). Physiological studies of carboplatin-treated chinchillas have confirmed that cubic distortion product otoacoustic emissions (DPOAEs) are generated by OHCs (Hofstetter et al., 1997a; Jock et al., 1996; Trautwein et al., 1996), and have led to the unexpected finding that thresholds of cochlear potentials and inferior colliculus potentials (ICPs) remain normal with moderate-to-large losses of IHCs as long as OHCs remain intact (Burkard et al., 1997; Jock et al., 1996; McFadden et al., 1998; Trautwein et al., 1996).

The mechanisms underlying the ototoxic effects of carboplatin are not known. However, many lines of evidence have implicated reactive oxygen species (ROS) in the general cytotoxicity of platinum compounds. Platinum cytotoxicity has been shown to be accelerated or enhanced in the presence of ROS, and decreased by antioxidants, particularly glutathione (GSH) (Ahn et al., 1994; Arrick and Nathan, 1984; Babu et al., 1995; de Graeff et al., 1988; Eastman, 1987; Ferguson, 1995; Lai et al., 1989; McGinness et al., 1978; Mistry et al., 1993; Russo et al., 1986; Tonetti et al., 1993; Walker and Gale, 1981). A number of studies have demonstrated correlations between GSH levels and sensitivity of tumor cells to platinum compounds (Ahn et al., 1994; Arrick and Nathan, 1984; Meijer et al., 1990, 1992; Mellish et al., 1993; Mistry et al., 1991). Cells with inherent or acquired resistance to platinum compounds have higher levels of GSH than susceptible cells, and depletion of cellular GSH can increase tumor cell sensitivity to platinum compounds.

Ravi et al. (1995) observed significant changes in antioxidant enzyme levels in the rat cochlea following cisplatin treatment: superoxide dismutase (SOD), catalase and malondialdehyde activity levels were increased, while GSH, GSH-peroxidase and GSH-reductase activity levels were decreased. Administration of diethyldithiocarbamate, which increases GSH and GSH-peroxidase activity but not SOD and catalase activity, significantly reduced threshold shifts in rats treated with cisplatin (Rybak et al., 1995). Collectively, these results suggest that GSH and GSH-dependent enzymes play a key role in platinum toxicity. Given the prominent role of GSH in the cellular antioxidant system and the fact that cochlear GSH is down-regulated with application of cisplatin (Ravi et al., 1995), it is reasonable to hypothesize that GSH may be a factor in determining the severity of carboplatin-induced cochlear damage as well.

L-Buthionine-[*S,R*]-sulfoximine (BSO) is a specific and irreversible inhibitor of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in the synthesis of GSH. BSO has been widely used as a tool to deplete intracellular and extracellular GSH in various cells and organs (Lee et al., 1987; Lee et al., 1992; Luthen et al., 1994; Mistry et al., 1993; Mitchell et al., 1989; Mizui et al., 1992; Morales et al., 1994; Pileblad and Magnusson, 1989; Thanislass et al., 1995), including the cochlea (Hoffman et al., 1988; Lazenby et al., 1988). The purpose of the present study was to determine if BSO enhances carboplatin ototoxicity in the chinchilla cochlea. The severity of carboplatin ototoxicity was assessed physiologically by measuring DPOAEs and ICPs, and anatomically by cytocochleograms showing IHC and OHC loss. Since previous studies have shown that carboplatin preferentially damages the IHCs in chinchilla, it was of interest to determine if BSO treatment would increase the amount of damage to the OHCs.

2. Materials and methods

2.1. Subjects and surgery for implanting recording electrodes

Fourteen adult chinchillas (450–600 g) with normal hearing served as subjects. In all subjects, BSO was applied to the right ears and the left ears served as controls. The animals were randomly divided into three groups, two carboplatin-treated groups and one drug control group. The single-dose carboplatin group ($n=5$) received BSO in the right ear, followed 3 days later by a single dose of carboplatin (25 mg/kg i.p.). The double-dose carboplatin group ($n=4$) received BSO in the right ear followed by two doses of carboplatin (25 mg/kg i.p. $\times 2$). The first dose was administered 3 days after the beginning of BSO treatment, and the second dose was administered 4 days after the first. Chinchillas in the drug control group ($n=5$) only received BSO in the right ear without any carboplatin. One animal in the drug control group and one animal in the single-dose carboplatin group failed to complete the experiment due to middle ear infections, leaving four animals (8 ears) in each group for data analysis.

Each subject was anesthetized with a mixture of ketamine (60 mg/kg) and acepromazine (0.5 mg/kg). Tungsten recording electrodes were stereotaxically implanted into the left and right IC and a ground electrode was implanted in the rostral cranium (McFadden et al., 1998). Following surgery, the animals were allowed to recover for at least two weeks prior to testing.

The care and use of animals in this study were approved by the State University of New York at Buffalo Institutional Animal Care and Use Committee.

2.2. Pump implantation surgery and carboplatin administration

BSO was infused into the right cochlea of each animal using an osmotic pump (2ML4, Alza Corporation) (Brown et al., 1993; Schindler et al., 1995). The pump was filled with 15 mM BSO (Sigma) in Hanks' balanced salt solution (Gibco). The pump infusion rate was 5 μl/h and the infusion period lasted 14 days. Our selection of a 15-mM concentration of BSO was based on previous in vitro studies, most of which used BSO concentrations ranging from 0.05 to 5 mM to deplete cellular GSH. We used a concentration of BSO exceeding the levels used in in vitro studies to allow for intracochlear dilution of the BSO solution by the perilymph. A 14-day pump was used to ensure that GSH levels were reduced over the entire time period that carboplatin was active.

The pump was prepared by connecting it to a catheter consisting of a short piece of polyethylene tubing. The distal end of the tubing was connected to a short piece of 27-gauge stainless-steel needle. A short piece of 33-gauge stainless steel tubing (Small Parts) was then inserted into the 27-gauge needle and cemented into place, with the tip protruding approximately 0.5 mm from the end of the 27-gauge needle.

During implantation, the animal was anesthetized with a mixture of ketamine (60 mg/kg) and acepromazine (0.5 mg/kg). The right bulla was exposed through a posterior approach and a small hole was made in the bulla in order to visualize the lateral wall of the first cochlear turn. A fine cutting needle was used to drill a small hole in the bony wall of the basal turn of the cochlea, allowing access to the scala tympani. The free end of the 33-gauge tubing was inserted into the scala tympani, and the shoulder formed by the 27-gauge needle butted up against the bony wall of the cochlea and limited insertion depth to 0.5 mm. A small ring of silicone glue was placed around the outside of the 27-gauge needle; this helped form a seal between the cochlea and the catheter. The catheter was fixed to the bulla with dental cement, the body of the osmotic pump was implanted under the skin on the back of the neck, and the incision was sutured closed.

Three days after implanting the osmotic pump, animals in the two carboplatin groups were injected with carboplatin (25 mg/kg, i.p., in 2 ml physiological saline). The animals in the double-dose group received a second dose of carboplatin 4 days after the first dose. Four drug control animals underwent the same BSO treatment, but were not treated with carboplatin.

2.3. ICP measurement

Tone burst stimuli were digitally generated (10 ms duration, 5 ms cosine rise/fall, constant starting phase, 10 stimuli/s) at 0.5, 1, 2, 4, 8 and 16 kHz as described previously (Hu et al., 1997). The stimuli were routed through a computer-controlled attenuator, buffer amplifier and insert earphone (Etymotic Research ER-2). Before each test, the sound pressure level (SPL) in the ear canal was measured from 100 Hz to 20 000 Hz with a probe tube microphone (Etymotic ER-7).

The electrical signal from the recording electrode was amplified (20 000×) and filtered (100–3000 Hz) by a Grass P511K amplifier. The output of the amplifier was digitized by an A/D converter on a digital signal processing board in a computer as described previously (Hu et al., 1997). Stimulus level was varied in 5-dB steps from 0 to 80 dB SPL. One hundred samples were averaged at each level. Thresholds were determined by visual inspection of raw waveforms at each frequency. Threshold was defined as the mid-point between the lowest level at which a clear response was seen and the next lower level where no response could be discerned. Mean thresholds were determined by averaging threshold estimates for all animals in a group. *I/O* functions show mean response amplitudes as a function of input level. The amplitude of the evoked response was measured from the first positive peak to the following negative trough. Thresholds and response amplitudes were measured before and 2, 7 and 14 days after the beginning of BSO treatment.

2.4. Distortion product otoacoustic emission (DPOAEs)

I/O functions of the cubic ($2f_1-f_2$) DPOAEs were recorded during the presentation of two primary tones (f_1 and f_2), with an f_2/f_1 ratio of 1.2. The primaries were generated by the D/A converter on two digital signal processing boards in a personal computer (Hu et al., 1997). The levels of the primaries, L_1 and L_2, were equal. DPOAE *I/O* functions were recorded in 5-dB steps from 0 to 80 dB SPL. DPOAE *I/O* functions were collected in random order for $f_1 = 1$, 2, 4, and 8 kHz. Average noise floor measurements were less than −10 dB SPL at all test frequencies. The chinchillas were placed in a custom-built restraining device (Snyder and Salvi, 1994) and DPOAEs were measured while the animals were awake. At least two sets of *I/O* functions were recorded prior to the BSO treatments and the average of these measures served as the baseline DPOAEs. DPOAEs were also measured 2, 7 and 13 days after the BSO treatment in the drug control group, and before and 14 days after the BSO treatment in the two carboplatin-treated groups.

2.5. Cochlear histology

Fourteen days after BSO treatment, the animals were anesthetized with sodium pentobarbital (50–100 mg/kg) and decapitated. Each bulla was quickly removed and

opened to expose the cochlea. After checking the bulla for signs of middle ear infection, the round window and oval window were opened and 0.2 M sodium succinate in 0.1 M phosphate buffer (pH 7.4) was slowly perfused through the round window; then the cochlea was immersed in the same solution for 1 h at 37°C. The cochlea was post-fixed with 10% formaldehyde for 24 h. Afterwards, the cochlea was dissected and sections of the organ of Corti were removed and mounted on a slide. The specimens were examined for HC losses under a light microscope (Zeiss Standard, 400×). The cytoplasm of surviving HCs was stained dark blue, while the areas of missing HCs showed an absence of staining. Hair cell counts were obtained over 0.24-mm intervals along the entire length of the cochlea and the percentage of missing OHCs and IHCs was plotted as a function of percent distance along the length of the cochlea as described previously (Hofstetter et al., 1997b; Hu et al., 1997).

3. Results

3.1. Effects of chronic BSO infusion

Data from the four animals that received BSO (15 mM) for 14 days without carboplatin were used to assess the effects of BSO infusion on cochlear function and morphology. Thresholds and response amplitudes of ICPs were examined before and at various times (2, 7, and 14 days) during the BSO infusion. Two days after beginning the BSO treatment, ICP thresholds increased by an average of 5–10 dB at tested frequencies. In one animal, the threshold shift at low frequencies reached 20–25 dB. Generally, thresholds recovered completely by 7 days after the beginning of BSO treatment, and were normal at 14 days.

Fig. 1 shows average ICP amplitudes at frequencies from 0.5 to 16 kHz, before and after 2, 7, and 14 days of BSO treatment. Note that 2 days after the beginning of BSO infusion, average ICP response amplitudes were decreased slightly, while at 7 and 14 days after BSO treatment, response amplitudes had returned to pre-treatment levels.

As with ICP *I/O* functions, DPOAE *I/O* functions obtained from the drug control subjects showed small amplitude losses 2 days after the beginning of BSO treatment, but normal amplitudes at 7 and 13 days (Fig. 2). At primary levels of 55 dB SPL, DPOAE amplitudes were decreased by 4.5–9.2 dB 2 days after the beginning of BSO treatment, but were normal by 7 days. OHC and IHC losses were assessed after 14 days

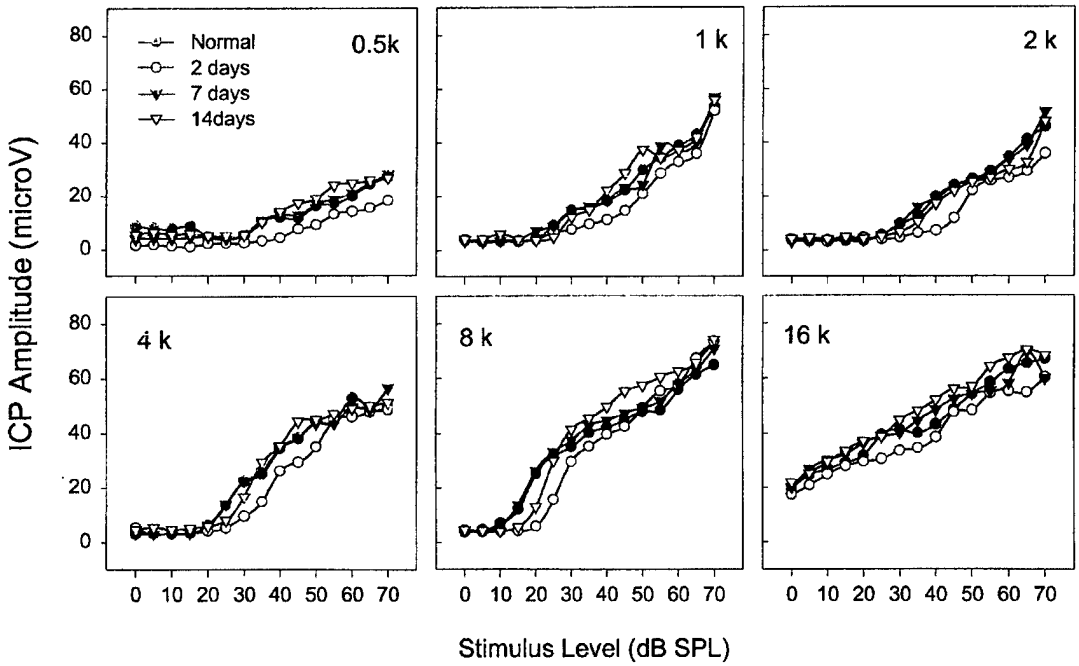

Fig. 1. Mean ICP amplitudes (μV, peak-to-peak) before BSO infusion (filled circles), and after 2 days (open circles), 7 days (filled triangles) and 14 days (open triangles) of BSO infusion in the drug control group. Each panel shows *I/O* functions for a different frequency (0.5–16 kHz). Minimum, maximum sds at each frequency were as follows. 0.5 kHz: 1.63, 5.43 (normal); 1.14, 7.04 (2 days); 1.11, 6.00 (7 days); 1.87, 8.65 (14 days). 1 kHz: 1.68, 7.08 (normal); 1.52, 8.30 (2 days); 0.67, 5.73 (7 days); 1.35, 4.82 (14 days). 2 kHz: 1.50, 9.51 (normal); 1.51, 5.32 (2 days); 0.84, 6.03 (7 days); 1.57, 6.07 (14 days). 4 kHz: 0.75, 5.86 (normal); 0.92, 6.11 (2 days); 1.31, 5.76 (7 days); 1.35, 7.69 (14 days). 8 kHz: 1.15, 8.03 (normal); 1.34, 7.06 (2 days); 1.53, 8.50 (7 days); 1.83, 6.82 (14 days). 16 kHz: 1.98, 8.77 (normal); 1.40, 8.04 (2 days); 1.40, 10.16 (7 days); 1.62, 8.57 (14 days).

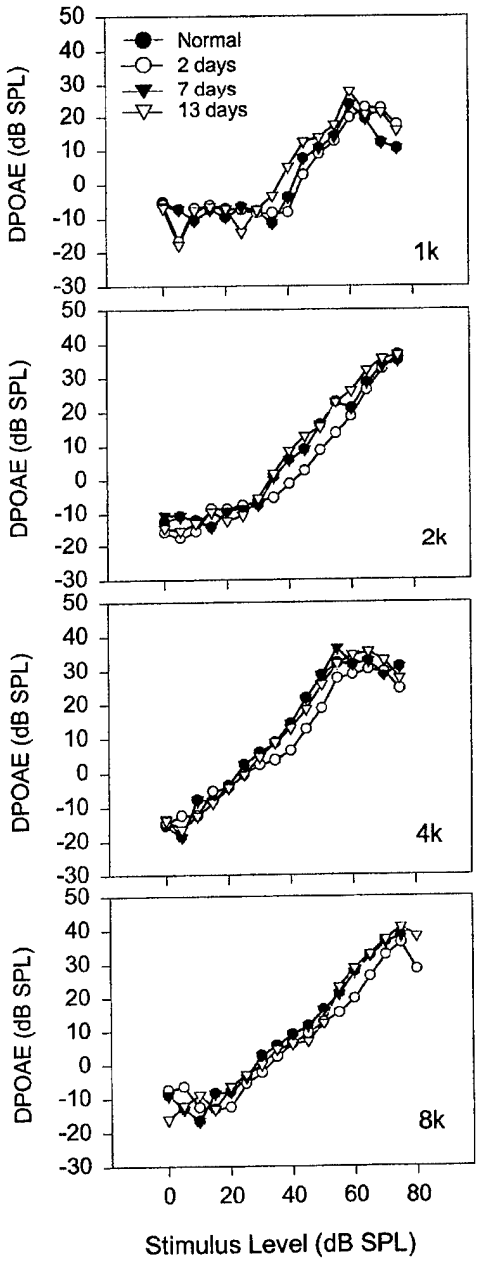

Fig. 2. Mean DPOAE amplitudes (dB SPL) measured before BSO infusion (filled circles), and after 2 days (open circles), 7 days (filled triangles) and 13 days (open triangles) of BSO infusion in the drug control group. Each panel shows *I/O* functions for different primary frequencies (f_1=1, 2, 4, and 8 kHz). Minimum, maximum sds at each frequency were as follows. 1 kHz: 1.43, 4.37 (normal); 1.07, 4.31 (2 days); 1.89, 3.61 (7 days); 1.28, 3.99 (13 days). 2 kHz: 1.24, 3.58 (normal); 1.63, 3.86 (2 days); 1.48, 4.92 (7 days); 1.66, 3.73 (13 days). 4 kHz: 1.60, 3.85 (normal); 1.15, 3.60 (2 days); 1.15, 4.18 (7 days); 1.54, 4.04 (13 days). 8 kHz: 1.66, 4.66 (normal); 1.61, 4.03 (2 days); 1.16, 3.95 (7 days); 1.47, 4.11 (13 days).

of BSO infusion. Light microscopic analysis of SDH-stained cochleas showed no signs of either IHC loss or OHC loss in any of the 8 cochleas from drug control animals.

3.2. Changes in ICPs, DPOAEs and HC losses after a single dose of carboplatin

Four animals were treated with a single dose of carboplatin (25 mg/kg) after 3 days of BSO pre-treatment. ICPs measured 11 days after carboplatin treatment (14 days after the beginning of BSO infusion) showed no significant changes relative to pre-drug measures, except for one animal in which ICP thresholds were elevated by 5–20 dB. As described below, histology revealed that this animal was the only one in the single-dose carboplatin group to show appreciable hair cell loss from carboplatin. There were no significant differences in mean DPOAE *I/O* functions between the control ears and the BSO-treated ears 11 days after carboplatin treatment, nor were there any significant changes relative to pre-drug measures.

Cochlear histology revealed that three out of the four chinchillas in the single-dose carboplatin group sustained relatively small IHC losses from carboplatin. As shown in Fig. 3 (upper panel), IHC losses in these three animals were confined to the cochlear region located 40–90% distance from the apex. The average IHC loss in the three BSO-treated ears was $5.76 \pm 6.0\%$, vs. $5.56 \pm 8.8\%$ for the control ears. A paired *t*-test con-

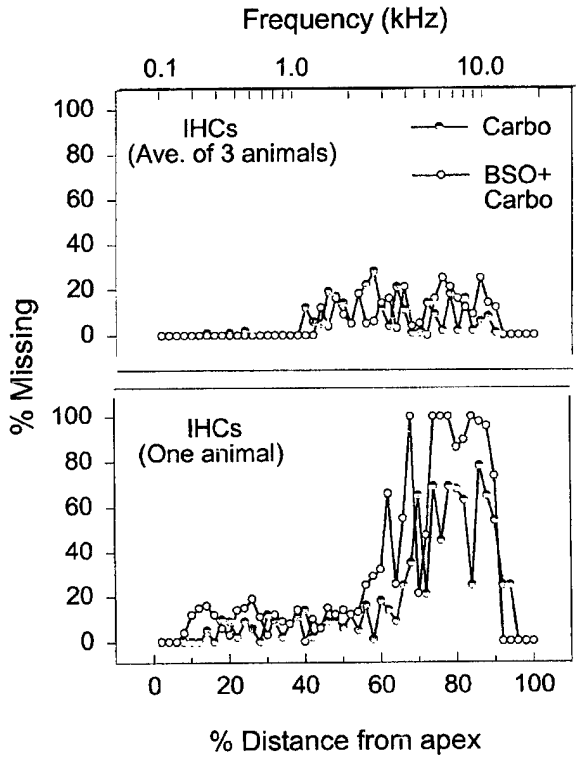

Fig. 3. Cochleograms showing percent of missing inner hair cells (IHCs) in cochleas of animals in the single-dose carboplatin group. Top panel illustrates average cochleograms from three subjects with relative minor IHC loss. Bottom panel shows the cochleogram from one subject with relatively severe IHC loss.

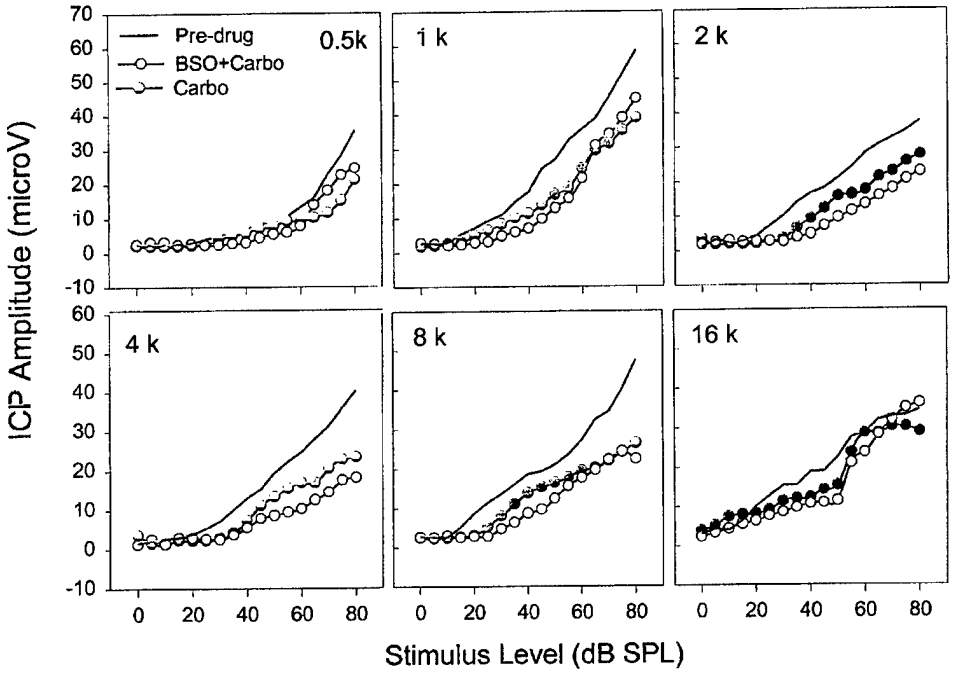

Fig. 4. Mean ICP amplitudes (μV, peak-to-peak) of animals in the double-dose carboplatin group, measured before and 14 days after the beginning of BSO infusion. Solid lines without symbols represent average of left and right ears before BSO infusion. Filled circles show amplitudes of control ears; open circles show amplitudes of the BSO-treated ears. Minimum, maximum sds at each frequency were as follows. 0.5 kHz: 0.93, 4.66 (Pre); 1.55, 4.27 (Ctr); 0.94, 7.51 (BSO). 1 kHz: 1.50, 6.20 (Pre); 1.55, 7.07 (Ctr); 1.52, 7.55 (BSO). 2 kHz: 1.33, 7.87 (Pre); 1.89, 4.91 (Ctr); 1.11, 5.44 (BSO). 4 kHz: 0.81, 5.76 (Pre); 1.14, 6.23 (Ctr); 1.00, 5.74 (BSO). 8 kHz: 1.13, 6.43 (Pre); 1.87, 7.11 (Ctr); 0.90, 8.17 (BSO). 16 kHz: 1.46, 5.33 (Pre); 0.96, 6.89 (Ctr); 1.60, 5.68 (BSO).

firmed that IHC loss in the BSO-treated ears was not significantly different from IHC loss in the control ears ($P = 0.87$). The fourth animal in the single-dose group sustained a large IHC lesion from carboplatin (Fig. 3, lower panel). This particular subject had approximately 30% greater IHC loss in the 60–90% region of the BSO-treated cochlea than in the control cochlea. The average IHC loss for this animal was $18.74 \pm 4.6\%$ in the control ear, vs. $29.97 \pm 2.74\%$ in the BSO-treated ear. A paired t-test confirmed that the average IHC loss in the BSO-treated ear of this animal was significantly greater than that in the control ear ($P < 0.001$).

Microscopic examination of OHCs showed little or no loss of OHCs in any of the four single-dose control ears, or in three of the four BSO-treated ears. The only cochlea with a notable loss of OHCs was the BSO-treated ear from the one animal with the large IHC lesion (described above). Even in this ear, however, average OHC loss across the cochlea was only 2%.

3.3. *Changes in ICPs, DPOAEs and HC losses after a double dose of carboplatin*

Fig. 4 compares the ICP *I/O* functions of BSO-treated ears and control ears of the four animals in the double-dose carboplatin group. The solid lines without symbols show the average *I/O* functions of right and left ears measured prior to BSO treatment, and the lines with symbols show *I/O* functions of BSO-treated ears (open circles) and control ears (filled circles) 7 days after the second dose of carboplatin (11 days after the first carboplatin injection). Both control ears and BSO-treated ears showed a significant decrease in response amplitude after carboplatin treatment. Amplitude losses tended to be slightly greater in BSO-treated ears than in control ears at stimulus levels between 45 and 60 dB SPL.

DPOAE *I/O* functions were also examined before BSO infusion and 7 days after the second dose of carboplatin application (Fig. 5). There was no significant change in amplitude after carboplatin treatment in the control ears. In contrast, BSO-treated ears exhibited decreased amplitudes at 2, 4, and 8 kHz following carboplatin treatments. At primary levels of 60 dB SPL, the BSO-treated ears had amplitude shifts of 4–12.3 dB relative to control ears and pre-drug values.

The double dose of carboplatin produced much greater IHC loss in both BSO-treated ears and control ears (Fig. 6, upper panel) compared to the single dose of carboplatin (Fig. 3). The magnitude of IHC loss varied across animals. However, all four BSO-treated ears had more IHC loss than their respective control ears. As shown in Fig. 6, IHC loss in control ears (solid circles) was localized to the middle and high frequency

regions of the cochlea, with no losses in the basal 90–100% region or in the apical 0–20% region. In contrast, BSO-treated ears had IHC losses scattered throughout all cochlear turns, with the greatest losses in the 60–80%

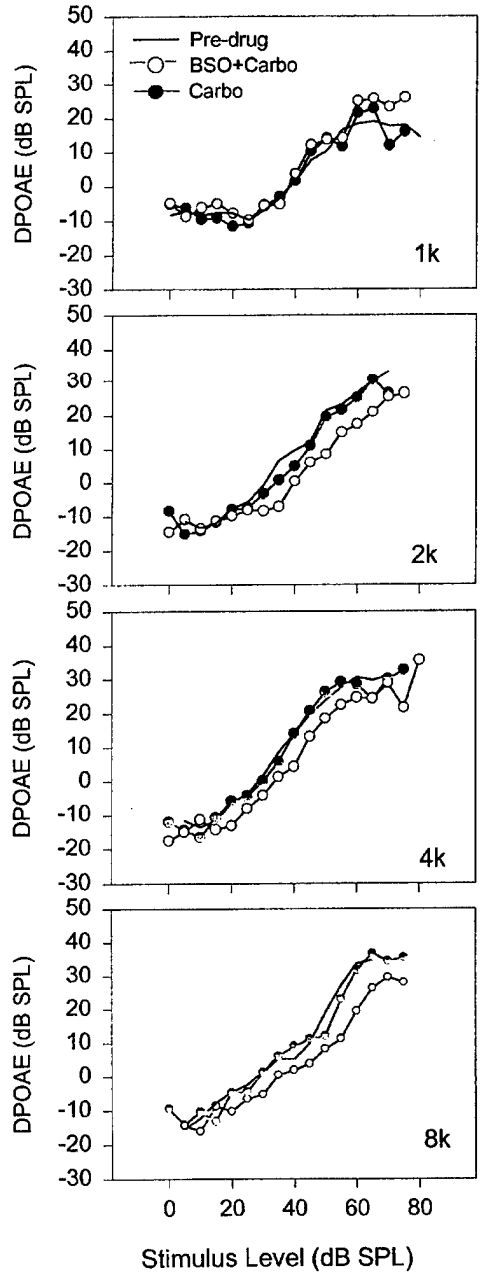

Fig. 5. Mean DPOAE amplitudes of animals in the double-dose carboplatin group, measured before and 14 days after the beginning of BSO infusion. Solid lines without symbols represent average of left and right ears before BSO infusion. Filled circles are for control ears; open circles are for BSO-pretreated ears. Minimum, maximum sds at each frequency were as follows. 1 kHz: 1.38, 3.89 (Pre); 1.16, 3.58 (Ctr); 1.72, 4.52 (BSO). 2 kHz: 1.66, 3.93 (Pre); 1.67, 4.04 (Ctr); 1.43, 3.56 (BSO). 4 kHz: 1.36, 4.05 (Pre); 1.39, 3.89 (Ctr); 1.24, 3.89 (BSO). 8 kHz: 1.02, 4.21 (Pre); 1.47, 4.24 (Ctr); 1.43, 4.03 (BSO).

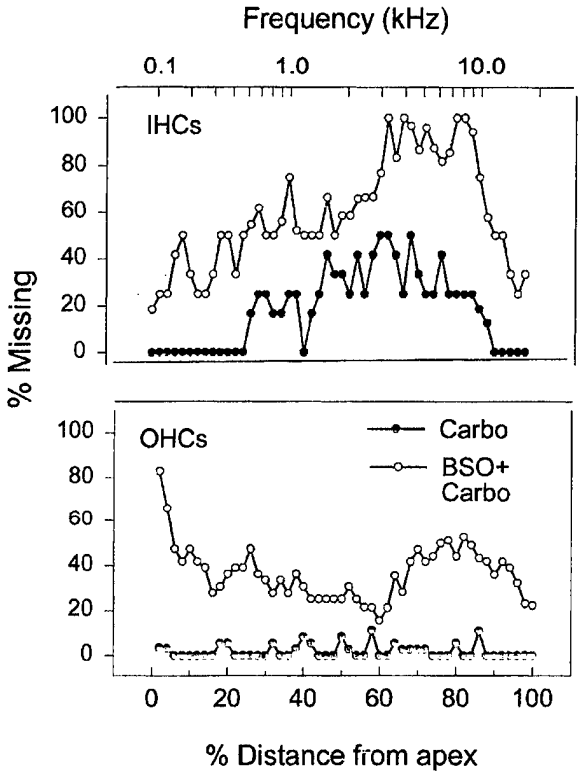

Fig. 6. Cochleograms for BSO-treated ears (open circles; n=4) and control ears (filled circles; n=4) following a double dose of carboplatin. Top panel shows average IHC loss; bottom panel shows average OHC loss.

region of the cochlea. Carboplatin produced an average IHC loss of 18.1 ± 4.62% in control ears and 59.1 ± 2.71% in BSO-pretreated ears. The difference between BSO-treated ears and control ears was statistically significant using a paired t-test ($P < 0.0001$).

The lower panel of Fig. 6 compares carboplatin-induced OHC losses in the BSO-treated ears (open circles) and control ears (solid circles). The control ears showed scattered OHC losses along the organ of Corti, with an average OHC loss of only 1.9 ± 8.8%. In contrast to the minor OHC losses in control ears, three out of the four BSO-treated ears had much greater OHC losses, and these losses were distributed along all turns of the cochlea. The remaining animal in this group had only minor OHC loss in the BSO-treated ear, which was similar to the OHC loss observed in the contralateral control ear. The average OHC loss for the group of four BSO-treated ears was 36.7 ± 6.0%. A paired t-test confirmed that average OHC loss was significantly greater in the BSO-treated ears than in control ears ($P < 0.0001$).

4. Discussion

The results show that infusion of BSO (15 mM) di-

rectly into the chinchilla cochlea potentiates the ototoxicity of carboplatin without causing direct damage to IHCs or OHCs. Potentiation of carboplatin ototoxicity was seen in one of four animals treated with a single low dose of carboplatin (Fig. 3), and in all four animals given two low doses of carboplatin, with a four-day interval between doses (Fig. 6). The differences between the BSO-treated ears and the control ears of animals in the double-dose carboplatin group were dramatic. Not only was there a significant potentiation of IHC loss, with BSO-treated ears showing approximately 41% more IHC loss than control ears, there was also a significant potentiation of OHC loss, with BSO-treated ears having an average OHC loss of approximately 37%, vs. 2% in control ears.

It is possible that potentiation of carboplatin damage was due to increased osmolarity from the Hanks' solution itself. Since osmolarity was not monitored and no blank control was used, this possibility cannot be ruled out. However, the strongest argument against increased osmolarity as the potentiator of carboplatin ototoxicity comes from other studies in our lab in which neurotrophins and protease inhibitors dissolved in Hanks' solution were pumped into the cochlea, leading to significant protection against ototoxicity and acoustic trauma (Salvi et al., 1998). The finding of protection in some cases and potentiation of damage in the present study argues that the substance dissolved in the Hanks' solution, not the Hanks' solution itself, is responsible for the effects. Since BSO is a specific and irreversible inhibitor of γ-GCS, potentiation of carboplatin damage is most likely mediated by inhibition of cochlear GSH synthesis (Kera et al., 1989; Kisara et al., 1995). The results therefore support a role of reactive oxygen species and GSH in carboplatin ototoxicity.

Two aspects of the results are particularly important to address in relation to hair cell vulnerability. The first issue is why BSO consistently potentiated IHC loss after a double dose of carboplatin but not after a single dose. The second issue concerns the clear potentiation of OHC loss in BSO-treated ears of animals in the double-dose group.

4.1. Potentiation of ototoxicity after a double dose of carboplatin

One question that arises from this study is why BSO consistently potentiated carboplatin ototoxicity in the double-dose carboplatin group but not in the single-dose group, despite the fact that BSO infusion parameters (dose, route, duration), and hence the degree of GSH depletion caused by BSO, were the same in both groups. The simplest explanation for this is that carboplatin treatment itself reduced cellular GSH, as has been shown to occur following cisplatin treatment (Ravi et al., 1995), and that the double dose of carboplatin, combined with BSO treatment, depleted GSH levels beyond a critical level. The fact that BSO infusion alone did not cause permanent anatomical or functional damage to HCs (Figs. 1 and 2) indicates that if GSH depletion is responsible for carboplatin damage, the effect is dose dependent. That is, permanent damage occurs only when the scavenging capacity of the antioxidant defense system falls below a critical level relative to ROS production. Since reduced activity of one antioxidant may be compensated by increased activity of a related antioxidant (e.g., decreased GSH activity may be compensated by increased catalase activity), the critical level for any given antioxidant will depend on many factors. Conceivably, the hypersensitivity of the one chinchilla in the single-dose carboplatin group could be an indication of a generally less efficient antioxidant defense system or a heightened production of ROS in this animal. In future studies, it will be useful to develop techniques for assaying cochlear levels of GSH and other antioxidant enzymes and correlating them with individual susceptibility to address these issues directly.

4.2. Potentiation of OHC loss

The most intriguing finding of this study was the potentiation of OHC loss in BSO-treated ears. Previous studies have clearly established that OHCs in the chinchilla are much less susceptible to carboplatin than IHCs. Hofstetter et al. (1997b) reported that the 'threshold' dose of carboplatin that produced significant OHC loss was about 200 mg/kg, approximately 5 times greater than that required to produce IHC loss. When OHC losses occur, they tend to progress along a base-to-apex gradient. In Hofstetter's studies, average OHC loss in the apical half of the cochlea was less than 20% for all carboplatin doses between 38 mg/kg and 200 mg/kg total. Average OHC loss in the basal half of the cochlea was less than 30% except at the highest (200 mg/kg) dose, when basal OHC loss climbed to 53%. In the current study, OHC loss was minor in all control ears. Consistent with this, DPOAEs, representing the functional status of OHCs (Trautwein et al., 1996), were completely normal. In contrast, BSO-treated ears of animals given a total carboplatin dose of only 50 mg/kg had OHC losses scattered throughout the cochlea, resulting in reduced DPOAE amplitudes at 2, 4 and 8 kHz.

The cellular mechanisms responsible for the greater sensitivity of IHCs to carboplatin in the chinchilla, the potentiation of both IHC and OHC damage by BSO, and the differences in susceptibility observed between chinchillas and guinea pigs are unknown. One possibility suggested by the present data is that levels of GSH and related antioxidant enzymes differ between IHC and OHCs. Since BSO enhanced the sensitivity of both IHCs and OHCs to carboplatin, presumably by

inhibiting GSH synthesis, it is reasonable to speculate that chinchilla OHCs are normally protected by higher endogenous levels of GSH than IHCs. To address this hypothesis, we have conducted pilot studies using mercury orange (1-4 chloro-mercury-phenyl-azo-2-naphthol; Sigma) to stain cellular thiols (the most prominent of which is GSH) in the cochlea. Preliminary results show that mercury orange staining is much more intense in the OHCs than in the IHCs. However, we have observed a similar pattern of staining in the guinea-pig cochlea, indicating that intracellular distribution of GSH cannot account for the species difference in HC susceptibility. Thus, species differences in HC vulnerability may be related to other factors, such as inherent differences in metabolic activity and ROS production, or differences in the ability of other antioxidants to compensate for decreased GSH production. More extensive experiments are required to understand cellular and species differences in susceptibility to carboplatin damage.

4.3. Clinical implications

Previous studies have shown that tumor cell sensitivity to platinum compounds is related to levels of GSH and GSH-dependent enzymes (e.g., Meijer et al., 1992; Mellish et al., 1993; Mistry et al., 1991). Inherent and acquired resistance to platinum drugs through GSH up-regulation poses an important problem for cancer treatment. Some studies have shown that decreasing cellular GSH levels by pre-treatment with BSO can increase the platinum sensitivity of some resistant tumor cell lines (Lee et al., 1992; Meijer et al., 1992; Mistry et al., 1991; Singh et al., 1995). However, results of the current study suggest that manipulating tumor sensitivity to platinum drugs through systemic BSO pre-treatment may potentiate ototoxic damage. Developing techniques for increasing the sensitivity of tumor cells to chemotherapy while avoiding increased sensitivity of cochlear hair cells and other tissues remains a challenge for both basic and clinical research.

Acknowledgments

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APPENDIX ID

Conditioning-induced protection from impulse noise in female and male chinchillas

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Sound conditioning (pre-exposure to a moderate-level acoustic stimulus) can induce resistance to hearing loss from a subsequent traumatic exposure. Most sound conditioning experiments have utilized long-duration tones and noise at levels below 110 dB SPL as traumatic stimuli. It is important to know if sound conditioning can also provide protection from brief, high-level stimuli such as impulses produced by gunfire, and whether there are differences between females and males in the response of the ear to noise. In the present study, chinchillas were exposed to 95 dB SPL octave band noise centered at 0.5 kHz for 6 h/day for 5 days. After 5 days of recovery, they were exposed to simulated M16 rifle fire at a level of 150 dB peak SPL. Animals that were sound conditioned showed less hearing loss and smaller hair cell lesions than controls. Females showed significantly less hearing loss than males at low frequencies, but more hearing loss at 16 kHz. Cochleograms showed slightly less hair cell loss in females than in males. The results show that significant protection from impulse noise can be achieved with a 5-day conditioning regimen, and that there are consistent differences between female and male chinchillas in the response of the cochlea to impulse noise. © *2000 Acoustical Society of America.* [S0001-4966(00)03404-4]

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INTRODUCTION

Pre-exposure to a moderate-level acoustic stimulus ("sound conditioning") can induce resistance to noise-induced hearing loss (NIHL). The protective effects of sound conditioning were reported first by Canlon *et al.* (1988), who found that guinea pigs that had been exposed to a 1-kHz tone at 81-dB sound pressure level (SPL *re*: 20 μPa) for 24 days incurred less permanent threshold shift (PTS) from a subsequent exposure to the tone at 105 dB SPL for 72 h than animals that had not been similarly "trained" or "conditioned." Since then, numerous studies have shown that sound conditioning can provide protection from NIHL in a wide variety of species and across a wide range of conditioning parameters. However, nearly all sound conditioning experiments have used long-duration tones or noise at levels below 110 dB SPL as their high-level stimuli. In four recent experiments, for example, high-level exposures consisted of an octave band noise (OBN) centered at 0.5 kHz at a level of 106 dB SPL for 48 h (McFadden *et al.*, 1997), an OBN centered at 1.4 kHz at 105 dBA for 3 days (Skellett *et al.*, 1998), an OBN centered at 2 kHz at 107 dBA for 48 h (White *et al.*, 1998), and a 6.3-kHz tone at 100 dB SPL for 24 h (Canlon and Fransson, 1998). These long-duration stimuli may induce cochlear damage by disrupting normal metabolic processes. In contrast, brief stimuli (impacts and impulses) at levels above 120 dB damage the cochlea by a combination of metabolic and mechanical processes (Hamernik *et al.*, 1984; Luz and Hodge, 1971). Many individuals develop NIHL as a result of exposure to impact noises in industrial settings and impulse noises produced by gunfire and explosions, particularly in the military. Therefore, it is important to know if sound conditioning can provide protection from brief, high-level noises as well as from continuous tones and noise.

Protection from impulse noise was reported by Henselman *et al.* (1994), who pre-exposed chinchillas to an OBN centered at 0.5 kHz at a level of 95 dB SPL for 6 h/day for 10 days. The animals were allowed to recover for 5 days, then they were exposed to impulse noise simulating M16 rifle fire at a level of 150-dB peak SPL. When assessed 30 days later, conditioned chinchillas had significantly less PTS and smaller hair cell lesions than control animals exposed to the impulse noise alone.

The present experiment has two primary aims. The first aim is to determine if significant protection from impulse noise can be achieved with a shorter conditioning regimen than that used by Henselman *et al.* (1994). The second aim is to determine if there are differences between female and male chinchillas in their response to impulse noise, or in their ability to develop resistance to NIHL through sound conditioning. This is an important issue to address in light of previous studies showing gender differences in susceptibility to NIHL in humans (Berger *et al.*, 1978; Gallo and Glorig, 1964; Ward, 1966) and sex differences in PTS in chinchillas exposed to impulse noise (McFadden *et al.*, 1999). In the latter study, female and male chinchillas were exposed to impulse noise at 150-dB peak SPL and PTS was assessed 30 days later. Female chinchillas developed approximately 10 dB more high-frequency hearing loss, but approximately 5 dB less low-frequency hearing loss than male chinchillas. On average, cochleas from females had 18% less inner hair cell (IHC) loss and 15% less outer hair cell (OHC) loss than males. The results suggested that there are sex/gender differences in the response of the cochlea to acoustic overstimulation that cannot be attributed to prior noise exposure history or other confounding factors.

I. METHODS

A. Subjects and surgery

Subjects were seven female and seven male adult chinchillas obtained from a commercial breeder (Jarr Chinchilla).

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Each subject was anesthetized with an intramuscular injection of ketamine hydrochloride (54 mg/kg) and acepromazine (0.64 mg/kg). A midline incision was made through the skin overlying the skull, and a small hole was made in the dorsal cranium overlying each inferior colliculus (IC). A recording electrode was advanced through the IC to a depth that produced a clear, large-amplitude response to an 80 dB SPL click, and cemented to the skull with cyanoacrylic adhesive and dental cement. A short tungsten electrode was implanted in the rostral cranium to serve as the common lead for evoked potential (IC-EVP) recording. Animals were allowed to recover for at least 10 days before testing. All procedures were reviewed and approved by the University of Buffalo Animal Care and Use Committee, and conformed to federal guidelines for the humane treatment of laboratory animals.

B. Evoked potential test procedures

The awake chinchilla was placed in a restraining device (Snyder and Salvi, 1994) in a foam-lined sound attenuating booth. Stimuli were digitally generated tones (2-ms rise/fall, 10-ms duration, 20/s rate) converted to analog signals by a 16-bit D/A converter on a digital signal processing (DSP) board in a personal computer. Stimuli were routed through a computer-controlled attenuator and impedance matching transformer to a loudspeaker (Realistic 401197) in the test booth. The speaker was located on the side of the animal's test ear, approximately 9 cm from the animal's pinna. The nontest ear was plugged with a foam insert earplug. Stimuli were presented in ascending order of frequency (in octave steps from 0.5 kHz to 16 kHz) and intensity (in 5-dB steps). Responses (electrical activity from the recording electrode in the IC contralateral to the test ear) were amplified (20 000×) and filtered (10–3000 Hz) by a Grass P511 bioamplifier and converted to digital signals by an A/D converter on a separate DSP board in the computer. Responses were computer averaged for 100 stimulus presentations. Threshold was defined as the mid-point between the level at which there was a clear deflection in the waveform and the next lower level at which there was none.

IC-EVPs were recorded (a) prior to noise exposure in order to establish pre-exposure baselines, (b) during the conditioning exposure in order to monitor temporary TS, (c) 5 days after conditioning to document TS recovery, (d) 15 min, 24 h, and 5 days after impulse noise exposure in order to monitor temporary TS, and (e) after 20 days recovery from high-level exposure to determine PTS. Pre-exposure and PTS measures for each animal represent the average of three tests performed on separate days. Threshold shifts were calculated by subtracting mean pre-exposure IC-EVP thresholds from post-exposure thresholds.

C. Noises and noise exposures

Animals were exposed to 0.5-kHz OBN for 5 days (6 h/day) at a level of 95 dB SPL, followed 5 days later by impulse noise simulating M16 rifle fire (Danielson *et al.*, 1991; Henselman *et al.*, 1994; McFadden *et al.*, 1999). The 0.5-kHz OBN was digitally generated, low-pass filtered (TDK HAF0030 active filter set at 20 kHz), manually attenuated (Hewlett Packard 350D), amplified (NAD 2200), and delivered to a compression driver (JBL 2446J) fitted to a bi-radial exponential horn (JBL 2360H). The driver/horn assembly was suspended from the ceiling of a sound booth. Animals were housed in separate cages (approximately 27 cm×21 cm×22 cm) placed beneath the loudspeaker, and provided free access to food and water during noise exposure. For acoustic calibration, sound pressure levels were measured with a calibrated Type I precision sound level meter (Larson-Davis 800B) and a 1/2-in. condenser microphone positioned at a height corresponding to the level of the ear canal of a standing chinchilla. SPL measurements were averaged across five positions within each cage (geometric center and each corner). Attenuator settings and cage positions were adjusted so that the average SPL in each cage was within 1 dB of the specified SPL. Animals were rotated to different cages each day to minimize any effects of slight differences in SPL between cages.

The impulse noise was generated digitally, converted to an analog signal by a 16-bit D/A converter on a DSP board, attenuated (Hewlett-Packard 350D), amplified (NAD 2200) and routed in parallel to two compression drivers (JBL 2446) in a sound booth. Each driver was fitted with a sound delivery tube (5 cm diameter×20 cm) with its end angled at 45° to broaden its range of resonance (Danielson *et al.*, 1991). Analysis of the impulse noise was performed with a Stanford Research Systems SR785 dynamic signal analyzer. The duration of the waveform was 29 μs, and the spectrum approximated a low-pass function with a half-power point at 400 Hz, rolling off at approximately 4 dB/octave between 400 Hz and 25.6 kHz.

The ends of the sound delivery tubes were separated by 10 cm. The animal in a restraining tube was placed between the drivers with the tubes directed at the animals' ears. Each animal was exposed to 50 pairs of impulses, with 50 ms between the two impulses of each pair and a 1000-ms interval between the onset of each pair. The total exposure time was 50 s. The impulse noise was calibrated using a 1/8-in. microphone (Bruel & Kjaer 4138) and voltmeter to a level corresponding to 150-dB peak SPL.

D. Cochlear analyses

After the completion of testing, chinchillas were deeply anesthetized with sodium pentobarbital (Somlethal, 400 mg/kg i.p.) and decapitated. The cochleas were quickly removed and perfused through the oval window with a succinate dehydrogenase (SDH) staining solution consisting of 2.5 ml of 0.2-M sodium succinate, 2.5 ml of 0.2-M phosphate buffer (pH 7.6), and 5 ml of 0.1% tetranitro blue tetrazolium. Cochleas were incubated in the SDH staining solution for 45 min at 37 °C, post-fixed with 10% formalin, and stored in fixative for at least 24 h. Stained cochleas were dissected from the apex to the base, mounted in sections in glycerin on microscope slides, coverslipped, and examined using light microscopy (400× magnification). The numbers of missing OHCs and IHCs were determined for successive segments of the organ of Corti. Individual cochleograms were constructed to show the percentage of hair cells missing

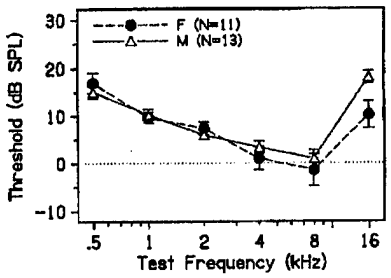

FIG. 1. Mean thresholds of female (solid circles) and male chinchillas (open triangles) before noise exposure. Bars in this and subsequent figures represent standard errors of the means (SEM).

as a function of distance from the apex of the cochlea. Percent hair cells missing was referenced to our lab standards based on normal chinchillas. Percent distance from the apex was converted to frequency using the frequency-place map of Greenwood (1990).

E. Data analysis

One male and two female chinchillas lost one or both IC recording electrodes during the experiment. Data from these ears were excluded from analysis. The final sample consisted of data from 11 ears of females and 13 ears of males. Data were analyzed using Statistical Package for the Social Sciences software (SPSS; version 8.0). An alpha level of 0.05 was adopted as the criterion level of significance for all statistical tests. Analyses of variance (ANOVAs) were used to assess differences between means, with IC-EVP thresholds and threshold shifts as dependent variables. Independent variables were Sex (a between-subjects factor), Frequency, and Time (within-subjects factors). Significant main effects and interactions involving Sex were analyzed further using independent Student *t*-tests. Changes as a function of time were assessed using paired *t*-tests. In order to determine if sound conditioning produced significant protection from impulse noise, data obtained from animals in this experiment were compared to data obtained from a control group of nine female and nine male chinchillas exposed to impulse noise alone under identical conditions, described in a previous publication (McFadden *et al.*, 1999). Comparisons between groups were made using separate two-way Group×Sex ANOVAs for mean low-frequency PTS (average of PTS values at 0.5, 1, and 2 kHz) and high-frequency PTS (average of PTS values at 4, 8, and 16 kHz).

II. RESULTS

A. Pre-exposure thresholds

Prior to exposure, females had lower threshold at 16 kHz than males (Fig. 1). A two-way (Sex×Frequency) mixed ANOVA showed a significant Sex×Frequency interaction, $F(5,110)=2.62$, $p=0.028$. The interaction occurred because females had similar or slightly higher thresholds than males at low frequencies, but consistently lower thresholds at high frequencies. The difference between females and males at 16 kHz was statistically significant [Student *t*-test, $t(22)=2.68$, $p=0.014$], with females having significantly lower thresholds than males. Pre-exposure thresholds were similar to those of control animals in our previous study (McFadden *et al.*, 1999). Control females also had significantly lower threshold at 16 kHz compared to control males, with no other significant differences between groups (Student *t*-tests).

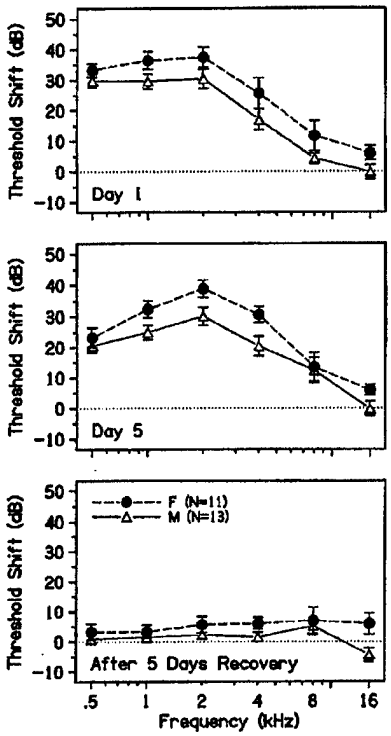

FIG. 2. Mean threshold shifts (±SEM) during and after sound conditioning. Top panel: threshold shifts after the first day (6 h) of sound conditioning. Middle panel: threshold shifts after the last day of conditioning. Bottom panel: threshold shifts 5 days after the last conditioning exposure and before exposure to impulse noise.

B. Threshold shifts from conditioning noise

After the first day of sound conditioning, thresholds were elevated by approximately 30–40 dB SPL at low frequencies, and by 0–25 dB at high frequencies (Fig. 2). Thresholds were significantly elevated at all frequencies for females and at all frequencies except 16 kHz for males (paired *t*-tests; all p values <0.04). Two-way (Sex×Time) mixed ANOVAs for TS at each frequency yielded significant main effects of Sex at 1, 2, 4, and 16 kHz [$F(1,22)=5.17$, 6.64, 7.02, and 8.35, respectively; all p values <0.04] and main effects of Time at all frequencies except 16 kHz (all p values <0.02). The main effect of Sex arose because females consistently showed more TS than males during and after the conditioning exposure.

TS decreased significantly at 0.5 kHz between Day 1 and Day 5 of conditioning for both males, $t(12)=2.84$, $p=0.015$, and females, $t(10)=2.97$, $p=0.014$. The decreases in TS that occurred between the last day of conditioning and the end of the 5-day recovery period were significant at frequencies below 16 kHz (all p values <0.04). After 5 days of

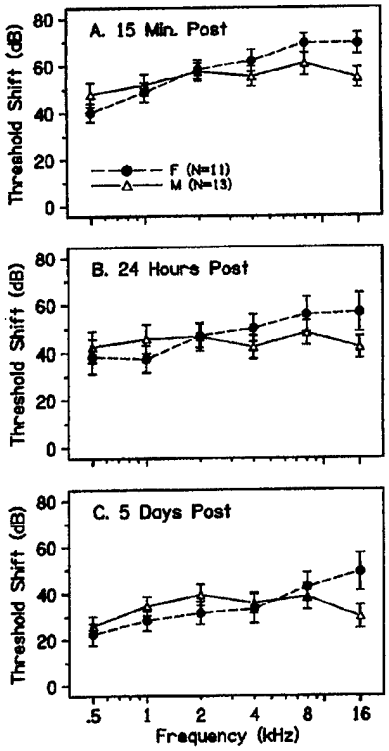

FIG. 3. Mean threshold shifts (±SEM) at three times after impulse noise exposure. Top panel: threshold shifts 15 min after impulse noise exposure. Middle panel: threshold shifts 1 day after impulse noise exposure. Bottom panel: threshold shifts 5 days after impulse noise exposure.

recovery from conditioning, thresholds were within approximately 5 dB of pre-exposure values at all frequencies (Fig. 2, bottom panel).

C. Threshold shifts after impulse noise

Mean TS values are shown in Fig. 3. Two-way (Sex×Time) mixed ANOVAs for TS at each frequency showed significant Sex×Time interactions at 2 and 4 kHz [$F(3,66)=5.36$ and 3.14, respectively, p values <0.04], and significant main effects of Time at all frequencies (all p values <0.001). The interactions occurred because females showed more recovery (approximately 10 dB) at 2 and 4 kHz

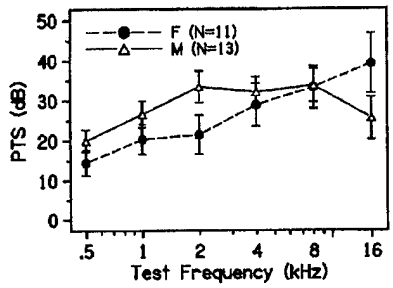

FIG. 4. Permanent threshold shifts (PTS) measured 20 days after impulse noise exposure for females (solid circles) and males (open triangles). Bars show SEMs.

over time than males. Between 15 min and 5 days post-exposure, TS decreased by approximately 20 dB for males, and 30 dB for females.

Impulse noise produced significant PTS at all frequencies (Fig. 4) for both males and females (paired t-tests; all p values ≤ 0.01). A two-way (Sex×Frequency) ANOVA on PTS showed a significant Sex×Frequency interaction, $F(5,110)=4.20$, $p=0.002$. The interaction occurred because of the reversal of sex differences at 2 kHz and 16 kHz. Males developed approximately 12 dB more PTS than females at 2 kHz, but approximately 15 dB less PTS than females at 16 kHz. Absolute thresholds of females and males at 16 kHz differed by less than 6 dB at 20 days post-exposure; this difference was not statistically significant.

To summarize the IC-EVP test results, females had a significantly lower mean threshold at 16 kHz than males prior to exposure (Fig. 1). During conditioning, females consistently showed greater TS than males, but thresholds of both sexes were essentially normal within 5 days after conditioning (Fig. 2). Subsequent exposure to M16 rifle fire produced more TS at low frequencies for males, and more TS at high frequencies for females (Fig. 3). When PTS was assessed 20 days after exposure to M16 rifle fire, females showed 6–12 dB less PTS than males at 0.5, 1, and 2 kHz, but approximately 15 dB more PTS than males at 16 kHz (Fig. 4).

D. Noise-induced hair cell losses

Hair cell lesions were slightly smaller in cochleas from females (Fig. 5). Average OHC losses in the apical half of

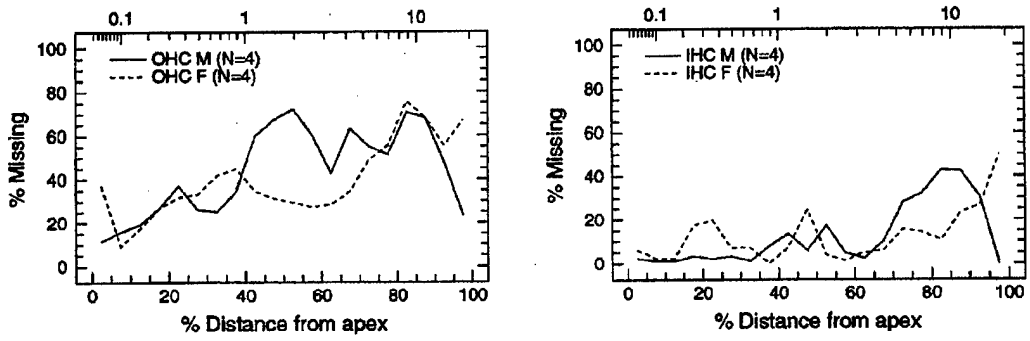

FIG. 5. Cochleograms showing hair cell loss after impulse noise exposure. Left panel: outer hair cell (OHC) losses for males (solid lines) and females (dashed lines). Right panel: inner hair cell (IHC) losses for males (solid lines) and females (dashed lines).

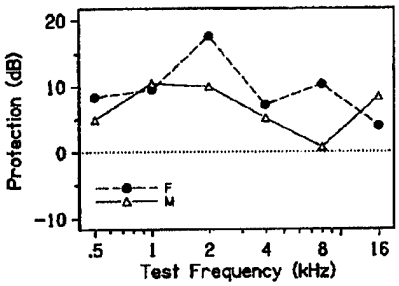

FIG. 6. Protection from permanent noise-induced hearing loss afforded by sound conditioning in females (solid circles) and males (open triangles). Protection is the difference in permanent threshold shift (PTS) between control animals exposed to the impulse noise alone and animals conditioned with 0.5-kHz octave band noise at 95 dB SPL for 5 days (6 h/day).

the cochlea were approximately 30% for females and 40% for males. Average OHC losses in the basal half were approximately 50% for females and 60% for males. The most striking difference was seen in the 2-kHz region of the cochlea, where males showed approximately 30% more OHC loss than females. Mean IHC losses were not remarkably different between females and males. Averaged across the entire cochlea, females had 11% IHC loss and 38% OHC loss, whereas males had 14% IHC loss and 47% OHC loss.

E. Protection from M16 rifle fire

One purpose of exposing animals to the 5-day conditioning regimen was to provide protection from subsequent exposure to M16 rifle fire. A perspective on the success of this approach is provided by Fig. 6, which shows differences in PTS between conditioned animals in the present study and control animals exposed only to the impulse noise (McFadden *et al.*, 1999). Sound conditioning provided up to 18-dB protection for females and up to 10-dB protection for males at individual frequencies. Collapsed across sex, the protective effect was 5–12 dB across frequencies, with greater protection at low frequencies than at high frequencies.

Figure 7 compares average low-frequency PTS and high-frequency PTS for conditioned and control animals. The pattern of PTS was similar for conditioned animals and controls. For both groups, females showed less low-frequency PTS but more high-frequency PTS than males. Conditioned females and males both showed less PTS than their same-sex controls. Two-way (Group×Sex) ANOVAs showed significant main effects of Group, $F(1,51)=6.70$, $p=0.012$, and Sex, $F(1,51)=4.70$, $p=0.035$, for low-frequency PTS. Thus conditioned animals showed significantly less low-frequency PTS than controls, and females showed significantly less low-frequency PTS than males. Despite the consistent trends for high-frequency PTS, neither differences between males and females nor differences between conditioned animals and controls were statistically significant.

Hair cell lesions were smaller in the conditioned animals compared to controls. Conditioned males had approximately 20% less IHC loss and 24% less OHC loss than control males. Conditioned females had approximately 5% less IHC loss and 18% less OHC than control females. Collapsed

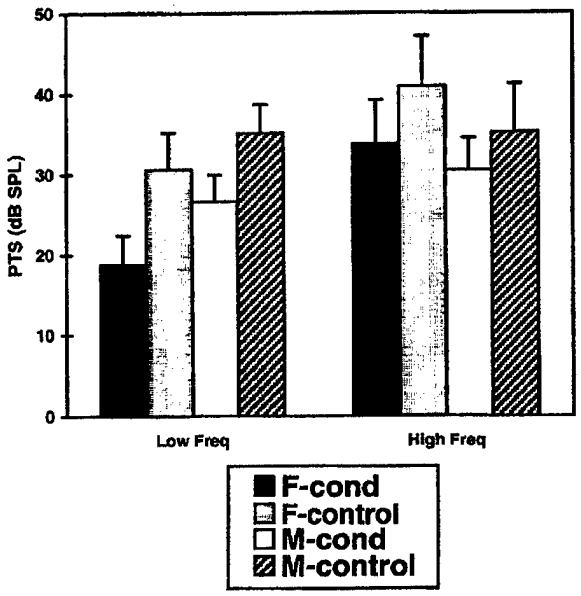

FIG. 7. Mean permanent threshold shifts (PTS) at low frequencies (average of 0.5, 1, and 2 kHz) and high frequencies (average of 4, 8, and 16 kHz) for conditioned females and males and their same-sex controls. Bars show SEMs.

across sex, sound conditioning resulted in approximately 12% less IHC loss and 21% less OHC loss compared to controls.

III. DISCUSSION

A. Protective effects of sound conditioning

The results show that sound conditioning provides protection from PTS and hair cell loss caused by impulse noise exposure. Chinchillas conditioned with a 0.5-kHz OBN at 95 dB SPL for 6 h/day for 5 days developed approximately 5–12 dB less PTS across frequencies, and 13%–21% less hair cell loss than chinchillas exposed to the impulse noise alone. As shown in Fig. 7, protection was apparent at high frequencies (approximately 6 dB overall) as well as low frequencies (approximately 10 dB overall). However, only the protection at low frequencies reached statistical significance.

The present results confirm and extend the results of an earlier study by Henselman *et al.* (1994) by showing that a 5-day conditioning regimen can protect the ear from impulse noise. A comparison between the two studies also shows a "dose effect" related to the duration of the conditioning exposure. In the Henselman experiment, a 10-day conditioning regimen produced approximately 7–23 dB of protection across frequencies, with the greatest protection (20–23 dB) at 2 and 4 kHz. Averaged across frequencies, the protective effect of a 10-day conditioning regimen was approximately 16 dB SPL. The 5-day conditioning regimen used in the present study also provided significant protection from PTS. However, the magnitude of protection was approximately 6–10 dB less than that provided by the longer conditioning exposure. Differences related to the duration of the conditioning exposure are also apparent in hair cell losses. The pattern of hair cell loss we observed in the present study was

similar to the pattern seen by Henselman *et al.* (1994). However, the magnitude of hair cell protection was considerably greater for the 10-day conditioning regimen. Animals conditioned for 5 days (present study) had approximately 40% OHC loss across the entire cochlea, whereas animals conditioned for 10 days (Henselman *et al.*, 1994) had less than 20% OHC loss. The dose effect of conditioning is interesting because it indicates that the magnitude of protection can be increased by extending the "training" period.

The mechanisms by which sound conditioning increase resistance to NIHL are not known, but they are clearly not "all or none" phenomena. Previous studies have indicated dose effects of sound conditioning related to exposure duration (Canlon and Fransson, 1998; Subramaniam *et al.*, 1993) and rest period between conditioning and high level exposure (McFadden *et al.*, 1997; Subramaniam *et al.*, 1992). In the experiment by Subramaniam *et al.* (1993), chinchillas were exposed to 0.5-kHz OBN at 95 dB SPL for 20 days, 10 days, or 2 times with 9 days between exposures. All three conditioning exposures provided protection from PTS, but the 10-day exposure provided the most protection overall. It is worth noting that similar protection was afforded by the 20-day conditioning exposure which resulted in residual TS and the two-time exposure that did not. The optimal "dosing" parameters for sound conditioning are difficult to determine, because the phenomenon depends on a complex interplay between species and stimulus variables (see McFadden and Henderson, 1999). Nevertheless, it is clear that exposure duration is an important dosing parameter to consider.

It is interesting that sound conditioning can protect the cochlea from impulse noise, which can damage the cochlea by causing direct mechanical failure as well as through metabolic disruption. Whether sound conditioning actually increases resistance to mechanical damage, or whether it only attenuates damage brought about by metabolic changes is not clear. However, there is some evidence that the structural components of the ear may be altered by sound conditioning in a way that could afford protection from mechanical damage (Hu and Henderson, 1997; Pack *et al.*, 1999).

B. Differences between female and male chinchillas

The results show significant differences between female and male chinchillas in their response to noise. During sound conditioning, females consistently showed greater TS than males. After impulse noise exposure, females developed approximately 10 dB less PTS at low frequencies, but approximately 5 dB more PTS at high frequencies than males. The difference in low-frequency PTS between females and males was statistically significant and is uncomplicated by preexisting threshold differences. The pattern of PTS in sound conditioned animals was very similar to that reported previously for animals exposed to impulse noise alone (McFadden *et al.*, 1999). In both cases, females had less TS at low frequencies and greater TS at high frequencies than males. Sound conditioning provided significant protection for both females and males, with little difference in the magnitude of protection.

The reasons for the sex differences cannot be determined from this study. However, since the differences were observed in chinchillas, extraneous factors such as differences in noise exposure history, recreational activities, and dietary factors can be ruled out. One possibility is that sex/gender differences in susceptibility to NIHL arise from acoustical properties of the outer and middle ears, as has been demonstrated for humans (Hellstrom, 1995). A second possibility is that sex/gender differences arise from basic physiological differences between female and male cochleas. Interestingly, Mills *et al.* (1999) recently reported that male rats are more susceptible to kanamycin ototoxicity than female rats, a difference that clearly cannot be attributed to acoustical properties of the ear. McFadden *et al.* (1998) observed changes in otoacoustic emissions in a human male treated with estrogen, indicating that sex hormones can influence outer hair cell function. Because the outer hair cell system is a likely candidate as a site for conditioning-induced changes (Canlon, 1996; Canlon and Fransson, 1995; Hu and Henderson, 1997), observations such as this may be important for understanding the sex differences we have observed in chinchillas. Future studies using the chinchilla may help determine the relative importance of anatomical and physiological factors in sex/gender differences in sound conditioning and susceptibility to NIHL.

ACKNOWLEDGMENTS

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APPENDIX IE

Differences Between Female and Male Chinchillas in Susceptibility to Noise-Induced Hearing Loss

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ABSTRACT

Small gender differences in auditory sensitivity have been well documented in humans. In addition, experimental studies of temporary threshold shifts (TS) and retrospective studies of hearing loss in industrial workers suggest that males are more susceptible to TS from low-frequency exposures, whereas females are more susceptible to TS from high-frequency exposures. Whether these differences are due to differences in noise exposure history, diet, and recreational activities or to inherent anatomic and/or physiological factors has not been determined. Furthermore, there is no information regarding gender differences in susceptibility to impulse noise. We conducted 6 separate experiments in which evoked potential thresholds were obtained from female and male chinchillas before and after exposure to noise. Each experiment utilized a different noise exposure condition: (1) 4 kHz octave band noise (OBN) at 105 dB SPL for 4 h; (2) impulse noise (simulated M16 rifle fire) at 150 dB pSPL with (a) a single driver positioned in front of the animal and (b) two drivers facing each ear and firing simultaneously; (3) 0.5 kHz OBN at 90-95 dB SPL for 5 days, followed by impulse noise; (4) noise simulating an Army Blackhawk helicopter at (a) 90 dB SPL for 5 days, followed by impulse noise, and (b) 112 dB SPL for 5 days, followed by impulse noise. The results show that prior to exposure, female chinchillas tend to have higher low-frequency thresholds and lower high-frequency thresholds than males. The differences are generally small (less than 5 dB SPL), but consistent across experiments. After exposure, males showed more low-frequency hearing loss (up to 15 dB) than females in all 6 experiments. Females showed more high-frequency hearing loss (up to 30 dB) than males in 4 experiments. Cochleograms generally showed greater hair cell losses in males than in females. Overall, the results suggest that there are sex differences in basic auditory sensitivity and in the physiological and anatomical response of the chinchilla cochlea to damaging levels of noise. Future studies with the chinchilla may be useful for understanding the nature of gender differences in humans.

Supported by DOD Grant DAMD17-96-1-6330 to SLM.

INTRODUCTION

Previous studies[1-9] have reported small but consistent gender differences in basic auditory sensitivity and susceptibility to noise-induced hearing loss (NIHL). In general, females tend to have slightly better pure-tone thresholds than males at frequencies above 1-2 kHz, while males may have slightly better thresholds below 1-2 kHz. Experimental studies have shown that males exhibit more temporary hearing loss (TTS) than females from low-frequency exposures (below 2 kHz), whereas females exhibit more TTS than males from high-frequency exposures (above 2 kHz). In an early investigation of gender differences in susceptibility to TTS produced by high intensity tones and noise, Ward (1966) conducted 17 experiments with 24 male and 25 female adults. Females showed approximately 30% less TTS than males when the exposure frequency was below 1 kHz, but approximately 30% more TTS when the exposure frequency was above 2 kHz. Most of what little we know about gender differences in permanent hearing loss (PTS) comes from retrospective studies of workers exposed to noise in industrial settings. Under these conditions, which typically involve exposure to low-frequency continuous noises, males tend to develop much more hearing loss (approximately 20 dB) than females.

The reasons for gender differences in auditory sensitivity and susceptibility to NIHL are unknown, but are generally attributed to systematic differences in the physical characteristics of the outer, middle and inner ears and/or noise exposure history. There have been no studies of sex differences in susceptibility to NIHL in non-human species to shed light on the issue. Here, we present data from six separate experiments conducted with chinchillas that suggest there are fundamental differences between female and male chinchillas in their auditory sensitivity and susceptibility to NIHL. All procedures were reviewed and approved by the University of Buffalo Animal Care and Use Committee, and conformed to federal guidelines for the humane treatment of laboratory animals.

METHODS

Subjects and Surgery. Chinchillas between 1 and 3 years of age were used. Recording electrodes were implanted in the left and/or right inferior colliculus (IC), and in the rostral cranium.

Test Procedures. Evoked potentials were recorded from the implanted electrodes before and 20-30 days after exposure to noise. The awake chinchilla was placed in a restraining device that held its head at a constant orientation within the calibrated sound field of a sound attenuating booth. Stimuli were 10 ms tones (2 ms rise/fall, 20/s rate) at octave intervals from 0.5 to 16 kHz. Threshold (dB SPL re: 20 μPa) was defined as the mid-point between the level at which there was a clear deflection in the waveform and the next lower level (5 dB step size) at which there was none.

Noise exposures. The noises were: (1) 4 kHz octave band noise (OBN) at 105 dB SPL for 4 hr (Exp. 1); (2) impulse noise simulating M16 rifle fire at 150 dB peak SPL (50 pairs; impulses in a pair spaced 50 ms apart; 1000 ms interval between the onset of each pair; 50 s total exposure time), using (a) a single driver positioned in front of the animal (Exp. 2) or (b) two separate drivers, facing each ear and firing simultaneously (Exp. 3); (3) 0.5 kHz OBN at 90-95 dB SPL for 5 days, followed by impulse noise (Exp. 4); (4) noise simulating UH60 Black Hawk helicopter noise at (a) 90 dB SPL for 5 days, followed by impulse noise (Exp. 5) or (b) 112 dB SPL for 10 days, followed by impulse noise (Exp. 6). Experiments 3-6 utilized two drivers for the impulse noise, and a 5-day rest period separated the first noise exposure from the impulse exposure. The spectra of the noises are shown in Figure 1.

Cochlear Analyses. Cochleas were stained with a succinate dehydrogenase (SDH) staining solution and post-fixed with 10% formalin. Surface preparations of the organ of Corti were examined with light microscopy. Cochleograms were constructed to show the percent of inner hair cells (IHCs) and outer hair cells (IHCs) missing as a function of location on the basilar membrane. In addition, mean cochlear length was determined for each group.

RESULTS

EXP. 1. 4 kHz OBN, 105 dB SPL, 4 Hours

Fig. 1. Pre-exposure thresholds and PTS of 4 females (8 ears) and 2 males (4 ears). Two-way mixed ANOVAs detected significant main effects of Frequency for both pre-exposure thresholds ($F(5,50)=21.25$, $p<0.001$) and PTS ($F(5,50)=10.97$, $p < 0.001$).

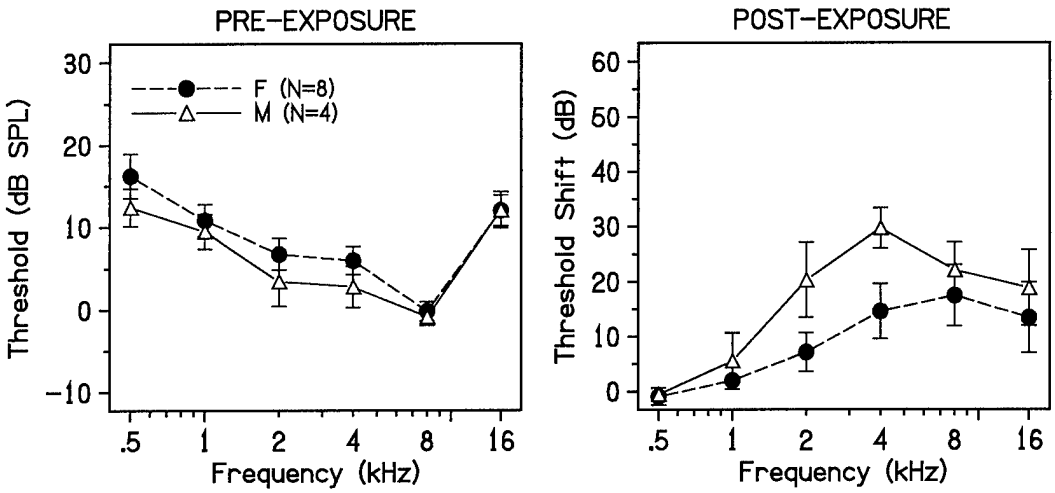
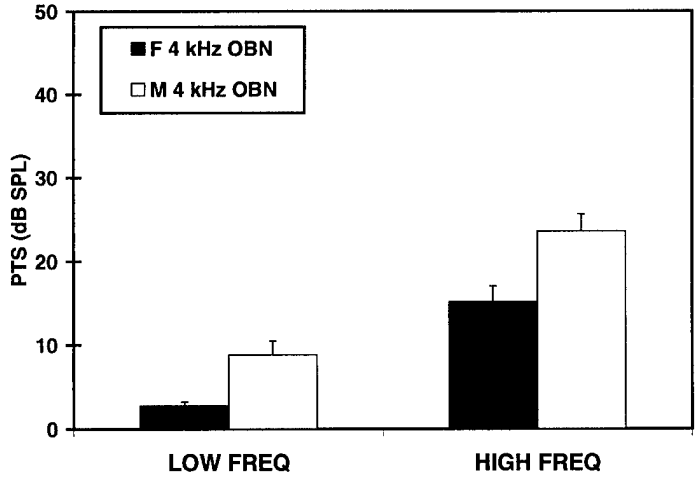


Fig. 2. Mean low-frequency (0.5, 1 and 2 kHz) versus high-frequency (4, 8 and 16 kHz) PTS.



EXP. 2. Impulse Noise, 150 dB pSPL, 50 pairs, 1 Driver

Fig. 3. Pre-exposure thresholds and PTS of 2 females (4 ears) and 5 males (9 ears). Two-way mixed ANOVAs detected significant main effects of Frequency ($F(5,55)=22.31$, $p<0.001$) for pre-exposure thresholds, and of Sex ($F(1,11)=6.48$, $p = 0.027$) and Frequency ($5,55)=6.34$, $p< 0.001$) for PTS.

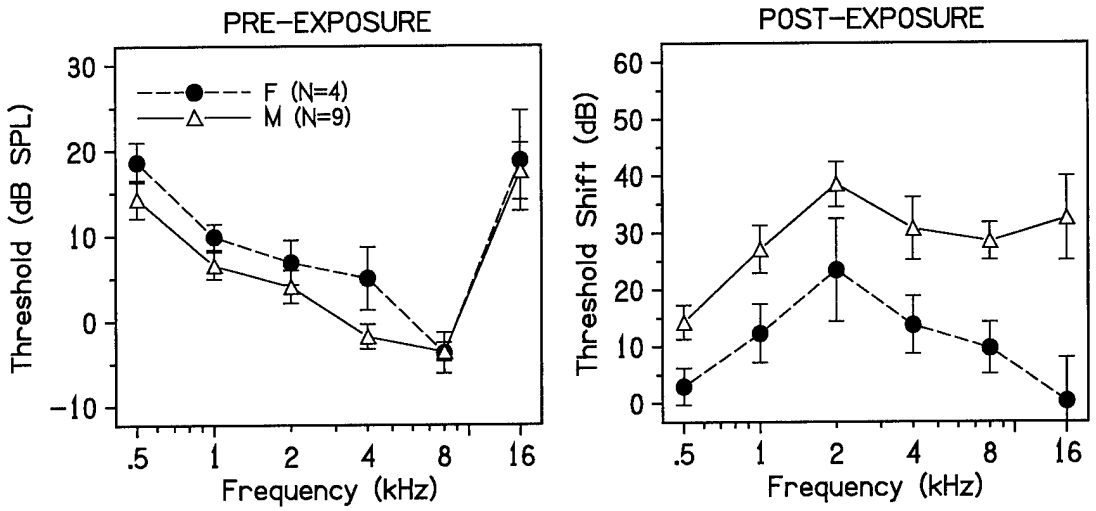


Fig. 4. Noise-induced OHC loss (left panel) and IHC loss (right panel). Mean cochlear length was 19.08 ± 0.4 for females, and 18.91 ± 0.9 for males.

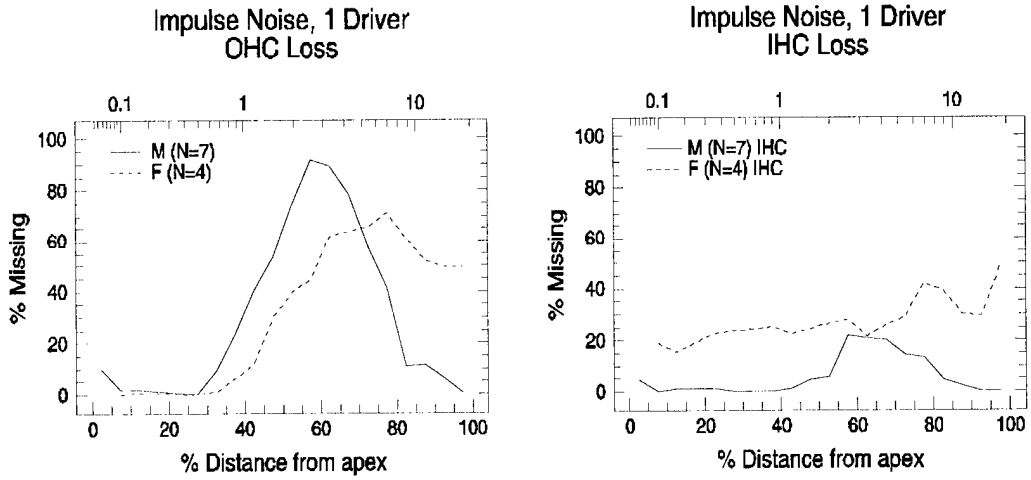
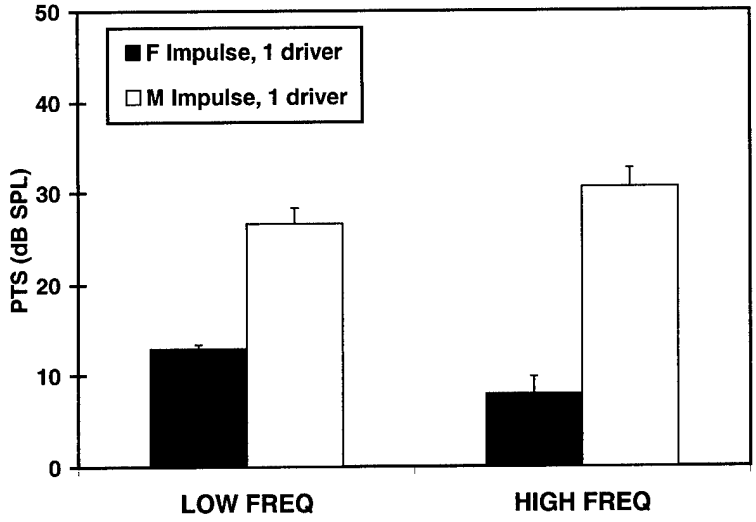


Fig. 5. Mean low-frequency versus high-frequency PTS.



EXP. 3. Impulse Noise, 150 dB pSPL, 50 pairs, 2 Drivers

Fig. 6. Pre-exposure thresholds and PTS of 9 females (15 ears) and 9 males (15 ears). Two-way mixed ANOVAs detected significant main effects of Frequency ($F(5,140)=47.07$, $p<0.001$) for pre-exposure thresholds, and of Sex ($F(1,11)=6.48$, $p = 0.027$) and Frequency ($F(1,28)=6.13$, $p = 0.02$) for PTS.

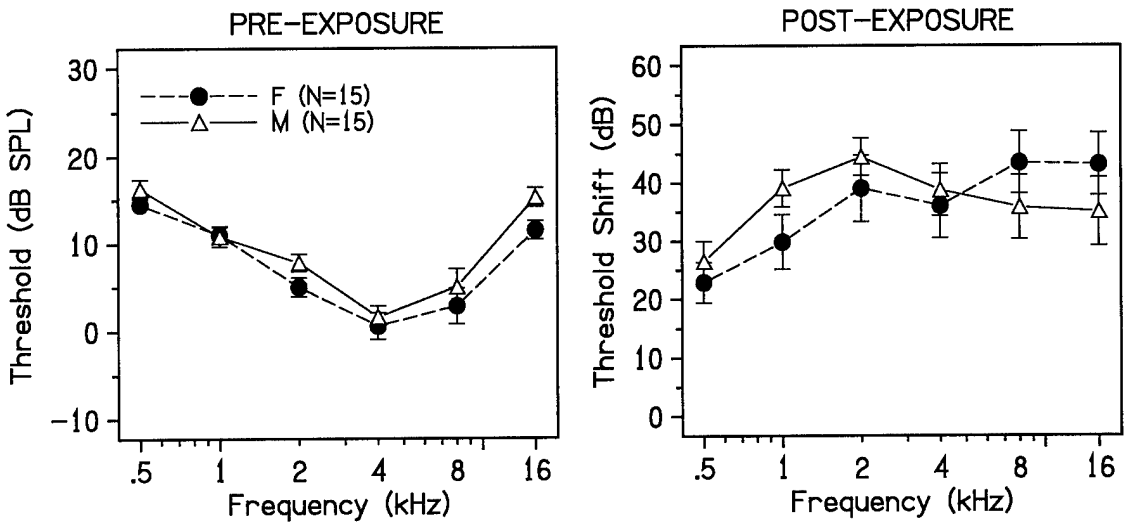
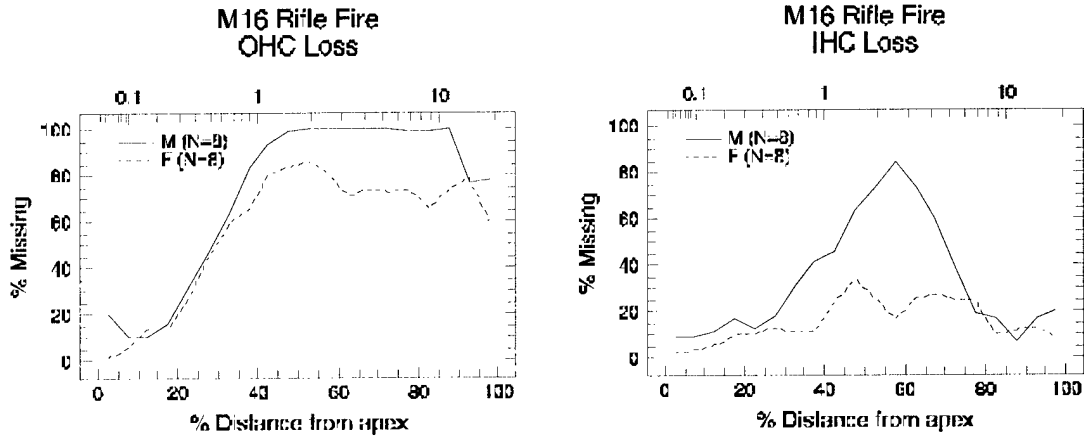


Fig. 7. Noise-induced OHC loss (left panel) and IHC loss (right panel). Mean cochlear length was 18.75 ± 0.8 for females, and 18.23 ± 0.6 for males.



EXP. 4. 0.5 kHz OBN, 90-95 dB SPL, 5 Days + Impulse Noise

Fig. 8. Pre-exposure thresholds and PTS of 6 females (11 ears) and 7 males (13 ears). Two-way mixed ANOVAs detected significant Sex X Frequency interactions for both pre-exposure thresholds ($F(5,110)=2.62$, $p=0.028$) and PTS values ($F(5,110)=4.20$, $p=0.002$), as well as main effects of Frequency ($F(5,110)=36.34$, $p < 0.001$ for pre-exposure thresholds, and $F(5,110)=8.45$, $p<0.001$ for PTS).

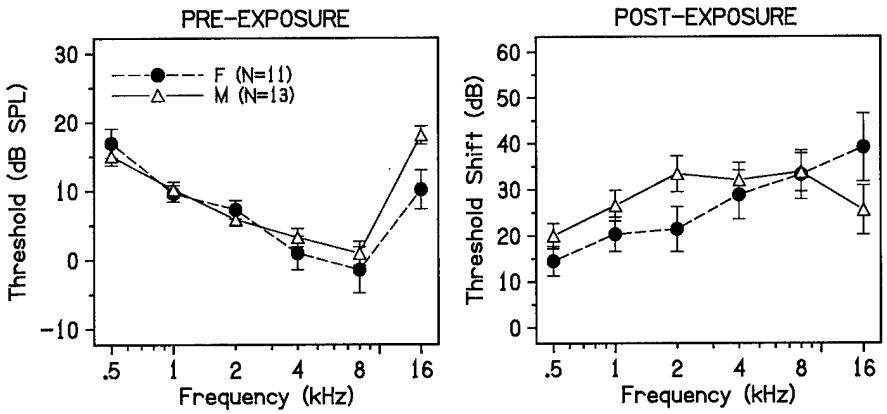
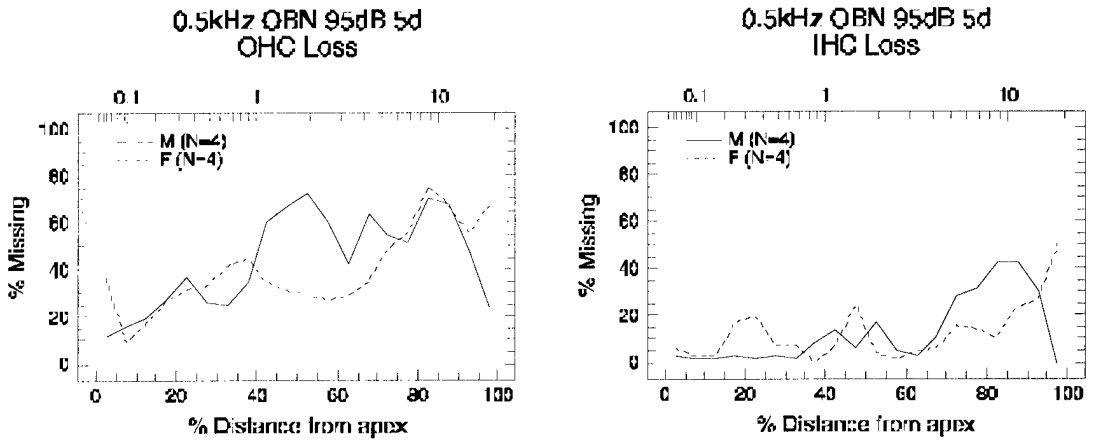


Fig. 9. Noise-induced OHC loss (left panel) and IHC loss (right panel). Mean cochlear length was 17.76 ± 0.9 for females, and 19.02 ± 0.5 for males.



EXP. 5. UH60 Helicopter Noise, 90 dB SPL, 5 Days + Impulse Noise

Fig. 10. Pre-exposure thresholds and PTS of 6 females (12 ears) and 6 males (12 ears). Two-way mixed ANOVAs detected significant Sex X Frequency interactions for both pre-exposure thresholds ($F(5,110)=2.36$, $p=0.045$) and PTS ($F(5,110)=2.45$, $p = 0.038$), as well as significant main effects of Frequency ($F(5,110)=86.63$, $p<0.001$ for pre-exposure thresholds, and $F(5,110)=16.27$, $p < 0.001$ for PTS).

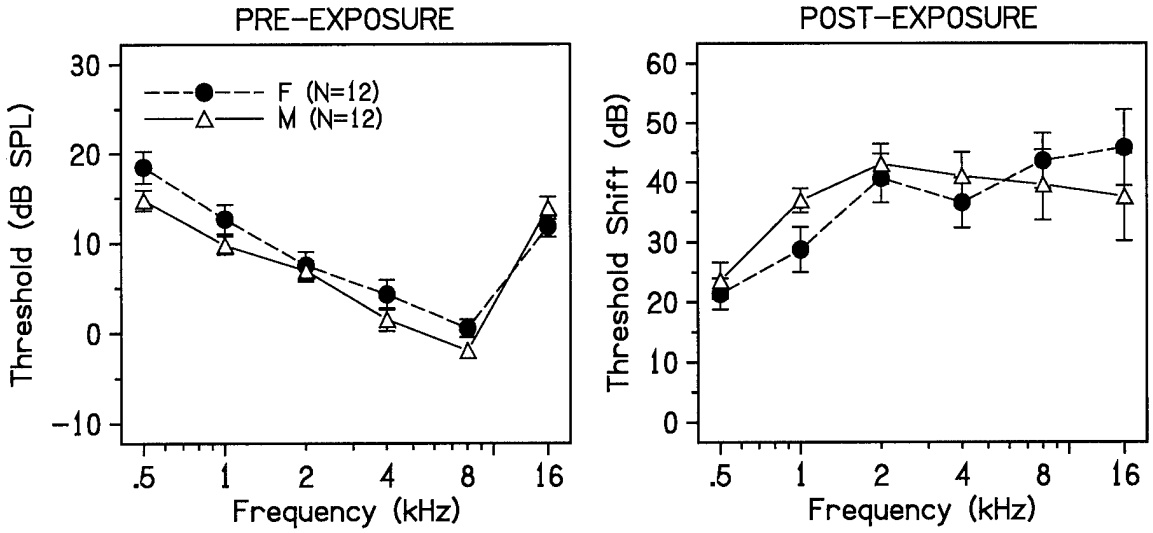
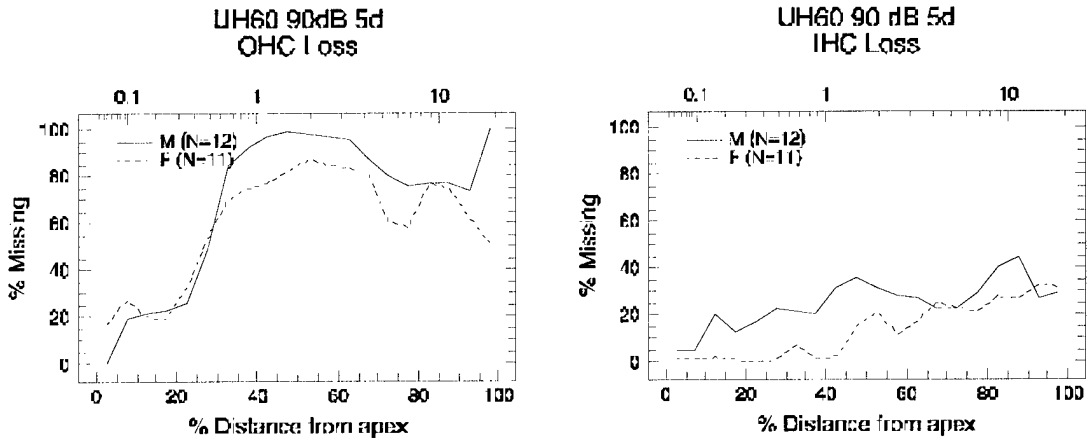


Fig. 11. Noise-induced OHC loss (left panel) and IHC loss (right panel). Mean cochlear length was 18.73 ± 1.2 for females, and 18.43 ± 0.5 for males.



EXP. 6. UH60 Helicopter Noise, 112 dB SPL, 10 Days + Impulse Noise

Fig. 12. Pre-exposure thresholds and PTS of 6 females (9 ears) and 6 males (9 ears). Two-way mixed ANOVAs detected significant Sex X Frequency interactions for both pre-exposure thresholds ($F(5,80)=2.89$, $p=0.019$) and PTS ($F(5,80)=3.81$, $p=0.004$), as well as main effects of Frequency ($F(5,80)=26.61$, $p<0.001$ for pre-exposure thresholds, and $F(5,80)=13.20$, $p < 0.001$ for PTS).

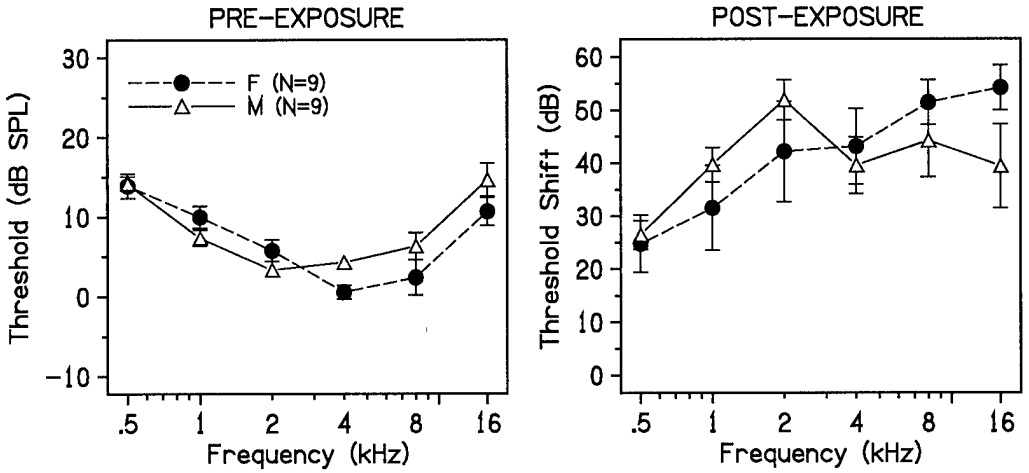


Fig. 13. Noise-induced OHC loss (left panel) and IHC loss (right panel). Mean cochlear length was 18.00 ±0.9 for females, and 18.96 ± 0.7 for males.

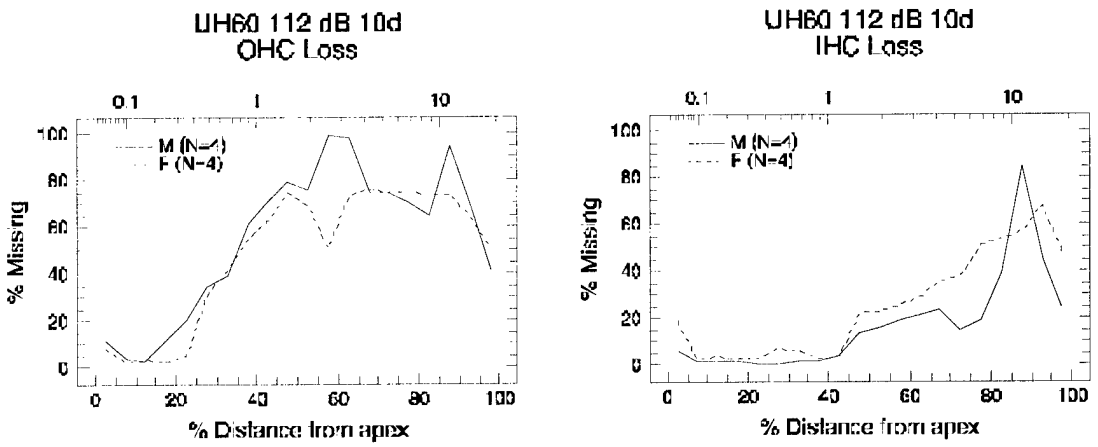


Fig. 14. Summary of Low-Frequency and High-Frequency PTS for Experiments 3-6.

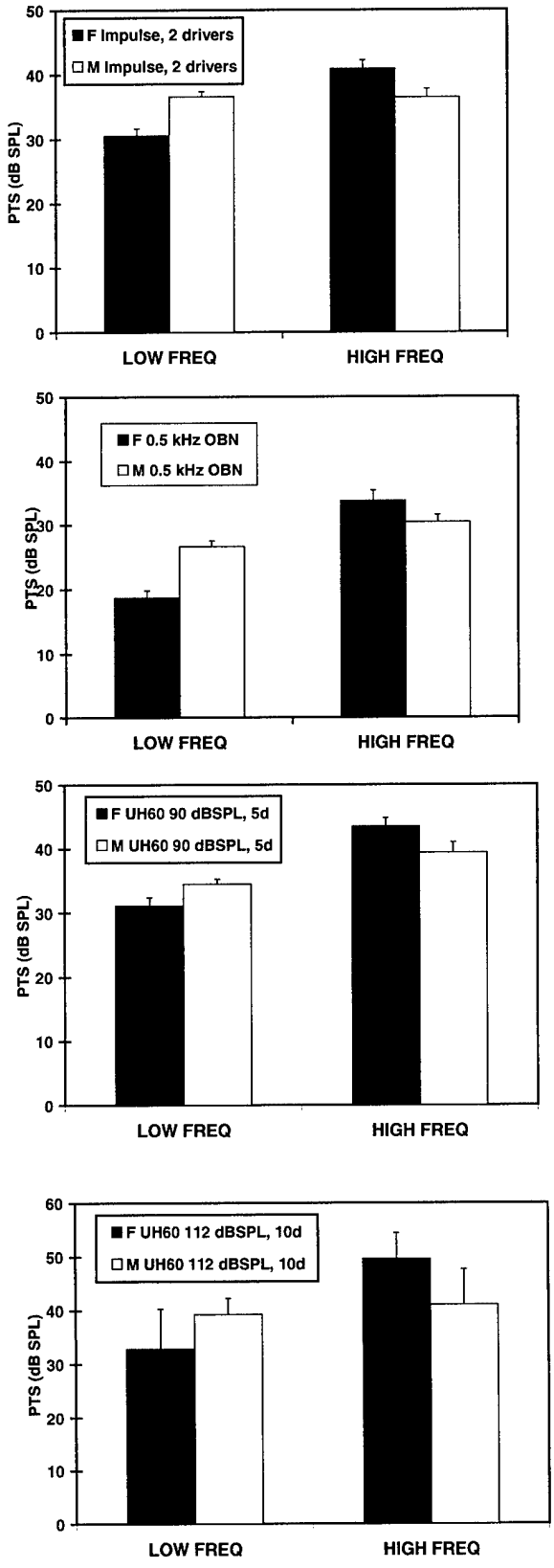


Fig. 16. Mean Length of Cochleas.

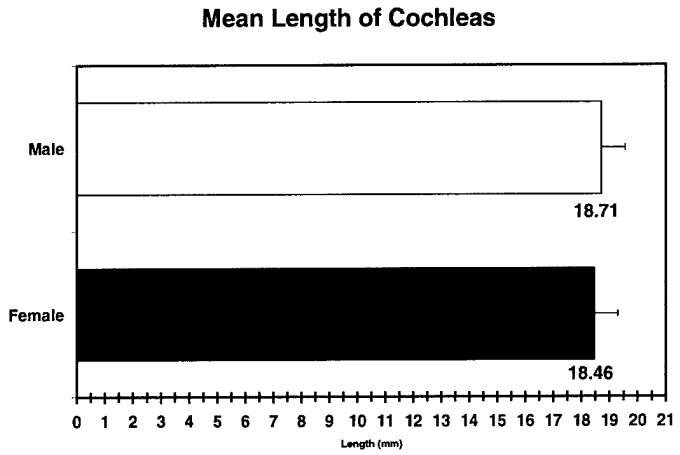
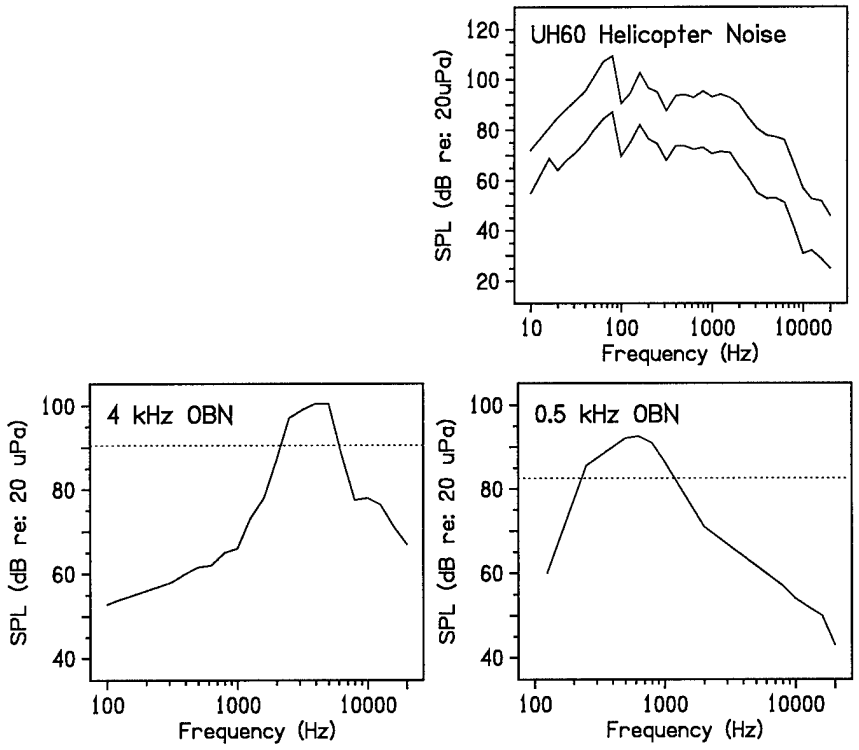


Fig. 16. Noise Spectra (1/3 Octave Band Level Measurements)



Summary and Conclusions

Female and male chinchillas differ slightly in their basic auditory sensitivity, with females tending to have lower thresholds at high frequencies and higher thresholds at low frequencies. More importantly, the results point to a fundamental sex difference in the response of the chinchilla cochlea to noise. Female chinchillas sustained more high-frequency hearing loss in 4/6 experiments, less low-frequency hearing loss in 6/6 experiments, and less hair cell loss in 6/6 experiments. The reasons for the sex differences cannot be determined from this study. However, since the differences were observed in chinchillas, they cannot be attributed to differences in noise exposure history, recreational activities, dietary factors, or other extraneous variables that complicate interpretation of gender differences in humans. Our measurements of cochlear length indicate that sex differences in susceptibility to NIHL are also not related to differences in the length of the cochlea in chinchillas.

Sex/gender differences in both basic sensitivity and in susceptibility to NIHL could arise from differences in the acoustical properties of the outer and middle ears[10-11], or from other as yet unknown physiological differences. Future studies using the chinchilla may help determine the relative importance of anatomical and physiological factors in sex/gender differences in auditory sensitivity and susceptibility to NIHL.

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APPENDIX IF

Systemic Treatment with Estradiol Reduces Noise-Induced Hearing Loss in the Chinchilla

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Association for Research in Otolaryngology
Midwinter Meeting
St. Petersburg Beach, FL
February 2000
Abstract 167, p. 48

ABSTRACT

The effects of estrogen (E) on noise-induced hearing loss were investigated in two experiments. Chinchillas were prepared for evoked potential (EVP) recording by implanting electrodes into each inferior colliculus (IC) and the rostral cranium. EVPs were obtained prior to noise exposure and at 15 min, 24 hr, 7 days and 14 days after exposure to 50 sec of impulse noise (Experiment I) or 4 hr of continuous noise (Experiment II). After baseline measurements were obtained, animals were randomly assigned to E or control groups. Animals in E groups received daily subcutaneous (s.c.) injections of 17-β estradiol (Sigma Chemicals) dissolved in olive oil. Animals in a vehicle control group received s.c. injections of olive oil on the same schedule as E animals. Animals in a separate control group received no treatment. In Experiment I, 10 animals received 200-265 μg E for 1-2 weeks prior to exposure. IC-EVP thresholds were not affected by E treatment. Following exposure to 50 pairs of impulses at 150 dB peak SPL, the E group showed significantly less hearing loss as compared to vehicle controls (N=4). In Experiment II, animals received either 100 μg (N=5) or 725 μg (N=6) E for 1 week prior to exposure. Following exposure to octave band noise centered at 4 kHz at 105 dB SPL, the E group showed significantly less hearing loss as compared to controls, and the high-dose group had less hearing loss than the low-dose group. The protective effects of E could be related to its antioxidant properties, its modulatory effects on neurotransmitter function, or to other properties of the steroid hormone.

Supported by Grant DAMD17-96-1-6330 to S.L.M.

INTRODUCTION

There is tremendous variability in the amount of hearing loss individuals develop from a given noise exposure. The reasons for individual differences in susceptibility to noise-induced hearing loss (NIHL) are largely unknown, but may include factors such as endogenous levels of antioxidant enzymes or steroid hormones. We have begun to examine the effects of "sex steroids" such as estrogen (E) on susceptibility to NIHL in chinchillas. Our preliminary results suggest that E can protect the ear from NIHL caused by both impulse noise and continuous noise. The mechanisms remain to be elucidated, but could involve alpha or beta E receptors that are abundantly expressed throughout the inner ear (Stenberg et al., 1999).

METHODS

Subjects and surgery: Adult chinchillas were anesthetized with ketamine and acepromazine. Tungsten electrodes were implanted into the inferior colliculus (IC) and the rostral cranium for recording auditory evoked potentials (EVPs).

EVP testing: An awake chinchilla was placed in a custom-designed restraint tube in a sound attenuating booth. Stimuli were 10 ms tones (2 ms R/F, alternating phase, 19-21/s rate) at 0.5, 1, 2, 4, 8 and 16 kHz. Stimulus level was incremented in 5 dB steps starting at a level below threshold. IC-EVP thresholds were measured twice before treatment, and at various times relative to treatment and noise exposure.

The following two figures provide a perspective on the reliability of IC-EVP amplitude functions. For individual animals, IC-EVPs are stable over time.

Figure 1. Test-retest reliability of pre-exposure IC-EVPs. The thin line represents the mean amplitude function on the first day of testing. The thick line shows the mean amplitude function 1-2 days later. The hatched region shows the 95% confidence interval for the group (n=5).

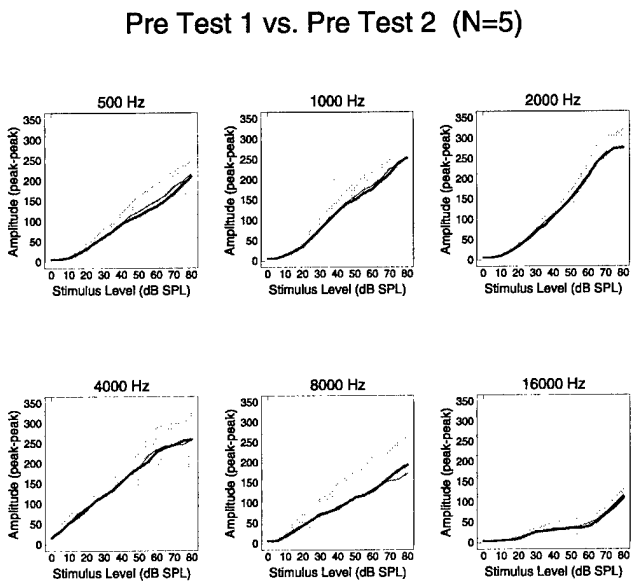
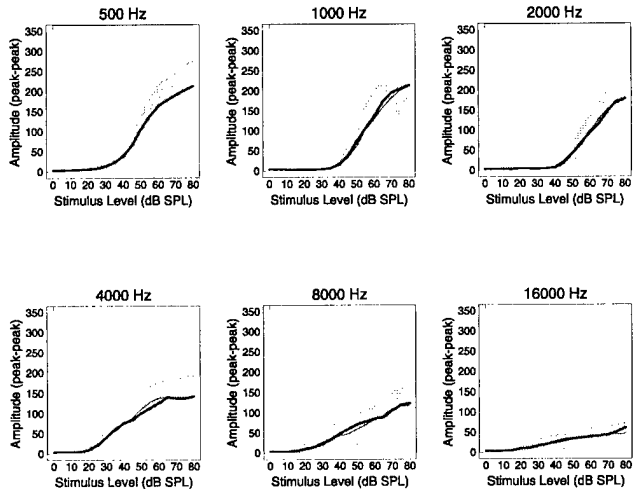


Figure 2. Test-retest reliability of post-exposure IC-EVPs. I/O functions from the same 5 animals shown above, measured 14 days (thin lines) and again 15-17 days (thick lines) after impulse noise exposure.



Noise stimuli and acoustic calibration: Animals were exposed to either impulse noise (150 dB peak SPL, 50 pairs, 1 s between pairs) that simulated impulses created by a U.S. Army M16A1 rifle, or octave band noise (OBN) with a center frequency of 4 kHz at 105 dB SPL for 4 h.

Groups and treatments: Animals in E treatment groups received daily s.c. injections of 17-β-estradiol (Sigma Chemicals) dissolved in olive oil vehicle. Animals in the **IMPULSE NOISE EXPERIMENT** were given E for 1-2 weeks before exposure, for total doses of **200 mg (n = 4) or 265 mg (n = 5)**. There were no differences between these two groups, so data were collapsed for analysis. Animals in the **4 kHz OBN EXPERIMENT** received E for 7 days prior to exposure. Total E dose was either **100 μg (N=5) or 725 μg (N=6)**.
Animals in **Control groups** either received equivalent amounts of vehicle via s.c. injection, or were untreated. There were no differences between vehicle-treated and untreated controls.

Cochlear histology: Cochleas were stained with a succinate dehydrogenase (SDH) staining solution, and post-fixed with 10% formalin. Cochleograms were constructed to show the percentage of hair cells missing as a function of distance from the apex of the cochlea, referenced to our lab standards for young adult chinchillas.

Estradiol assays: Blood samples were collected from 5 female and 4 male chinchillas. Samples were centrifuged and treated with a steroid displacement reagent to free estradiol bound to transport proteins in the serum. Estradiol levels were measured using an enzyme immunoassay kit from Assay Designs Inc. All samples were run in triplicate.

Data analyses:
One-way ANOVAs and Tukey tests were used to assess differences between means (IC-EVP thresholds, threshold shifts, hair cell loss). All statistical tests were evaluated using a 0.05 criterion of significance.

RESULTS

I. ESTRADIOL ASSAYS

Table 1. Levels of estradiol in picograms/ml serum. For each subject, the value listed is the average of 3 assays.

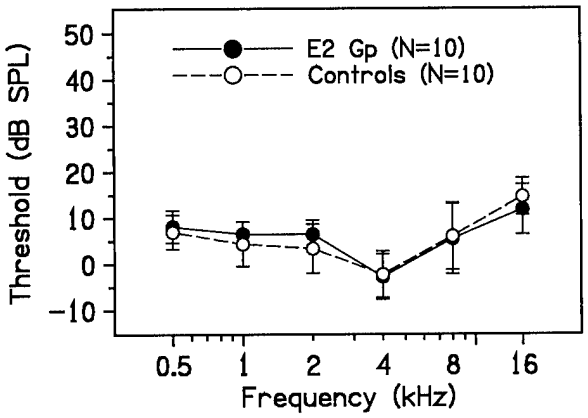
FEMALES	MALES
109.01	40.82
256.58	66.92
258.36	69.98
263.92	82.40
312.76	
MEAN = 240.12; sd= 76.87	**MEAN = 65.03; sd = 17.47**

Two aspects of these preliminary assay results are particularly interesting. First, the levels for female and male chinchillas are in the range reported for humans. Second, females have higher E levels and are much more variable than males. Because individuals show a wide range of variability, it will be possible in future studies to correlate endogenous levels of E with susceptibility to NIHL in both treated and untreated populations.

II. IMPULSE NOISE EXPERIMENT

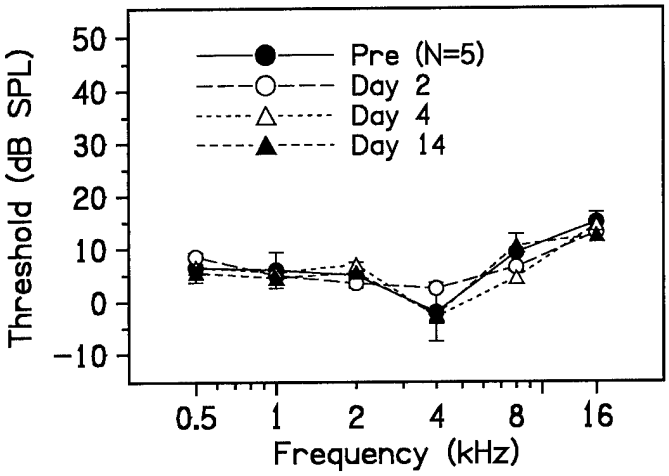
1. Thresholds of controls and E-treated chinchillas were not significantly different prior to exposure.

Figure 3. Thresholds (mean ± sd) prior to impulse noise exposure. There were no significant differences between groups at any frequency.



2. E treatment did not alter IC-EVP thresholds.

Figure 4. Thresholds (mean ± sd) measured after 2, 4 and 14 days of E treatment (≈20 μg/day; n = 5). Thresholds did not change significantly over the E treatment period, indicating that short-term E treatment has no direct effect on auditory sensitivity as measured by IC-EVPs.



3. E treatment provided protection at high frequencies.

Figure 5. Thresholds (mean ± sd) measured immediately (day 0), 7 days, and 14-21 days after exposure. Thresholds of E-treated chinchillas were consistently lower (by 10-20 dB SPL) than those of controls at 4, 8, and 16 kHz. However, only the difference at 16 kHz on day 14 was statistically significant.

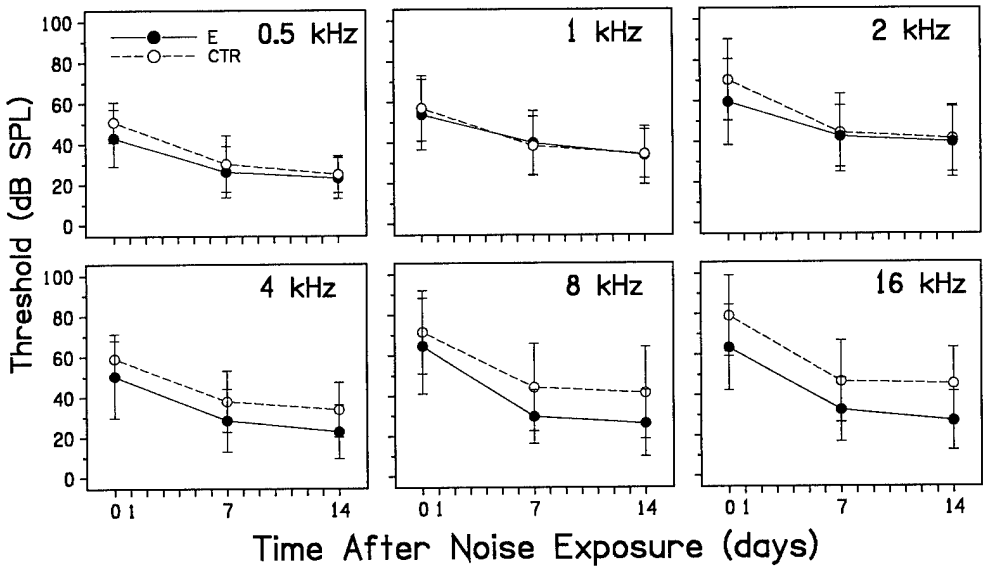
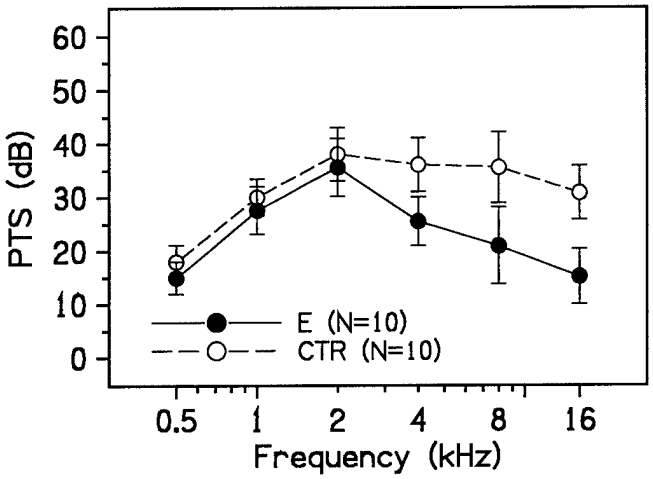
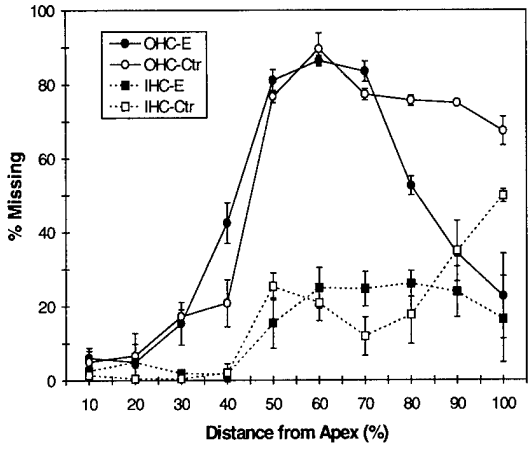


Figure 6. Permanent threshold shifts (mean ± SEM) after impulse exposure. The control group had PTS ranging from approximately 20 dB at 0.5 kHz to 40 dB at 2 kHz. The E-treated group had similar PTS at 0.5, 1 and 2 kHz, but consistently less PTS (by approximately 15-20 dB) at 4, 8 and 16 kHz. However, only the difference at 16 kHz was statistically significant.



4. E treatment protected hair cells in the extreme base of the cochlea.

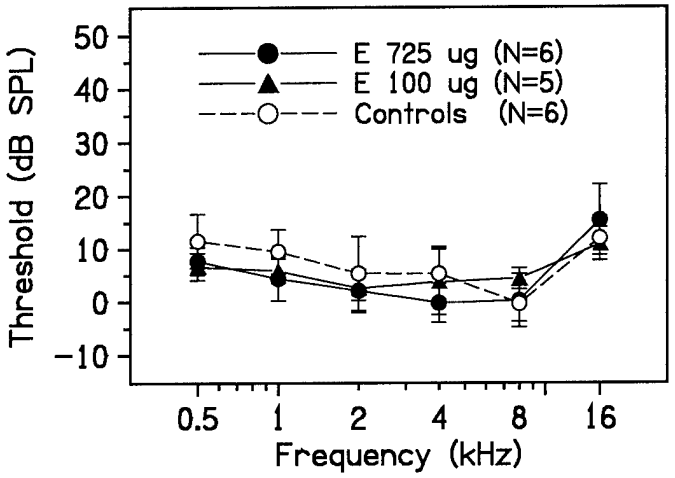
Figure 7. Hair cell losses (mean ± sd). E-treated animals had less hair cell loss than controls in the basal-most 10-20% of the cochlea. Differences in other regions were minor. Ctr: Control group (n = 4); E: Estradiol-treated group (N = 10).



IV. 4 kHz OBN EXPERIMENT

1. Thresholds of controls and E-treated chinchillas were not significantly different prior to exposure.

Figure 8. Thresholds (mean ± sd) prior to impulse noise exposure. There were no significant differences among groups at any frequency.



2. E treatment provided protection at high frequencies.

Figure 9. Threshold shifts (mean ± SEM) measured immediately (day 0), 1, and 7 days after exposure. There were no significant differences among groups at 15 min or 24 h after exposure. At 7 days post-exposure, animals treated with 725 mg E had significantly lower thresholds and less TS than controls or animals treated with 100 mg E at 8 kHz and 16 kHz.

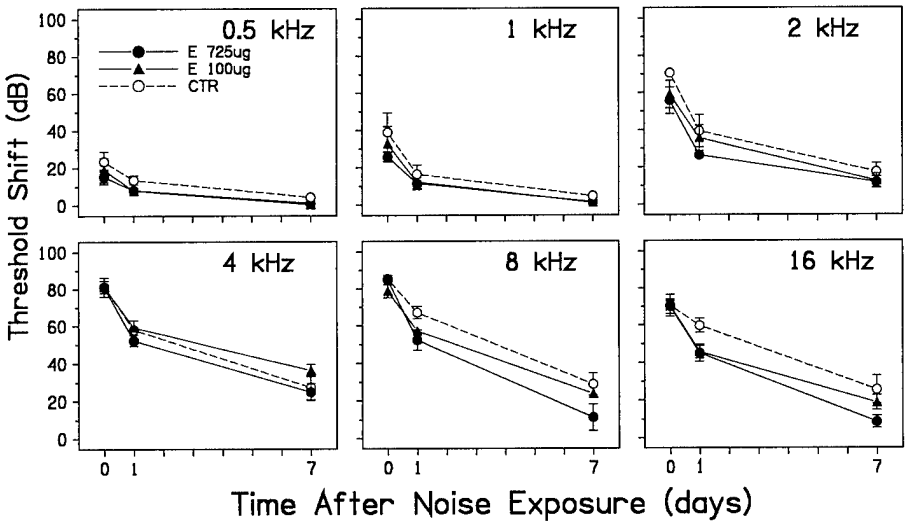
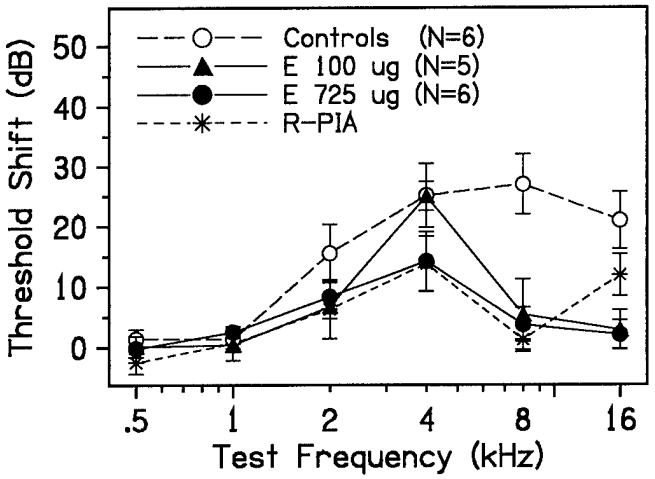


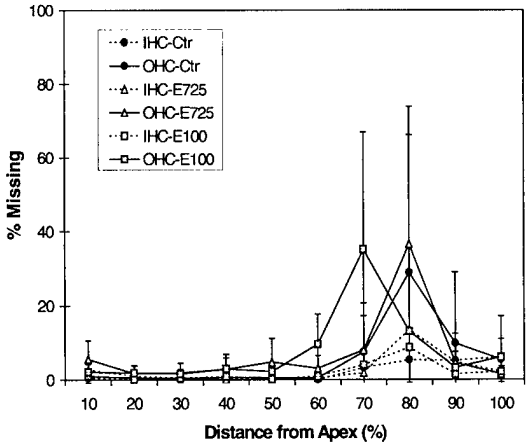
Figure 10. Permanent threshold shifts (mean ± SEM) 14-21 days after 4 kHz OBN exposure. Estradiol-treated animals had significantly less PTS than controls at 8 kHz and 16 kHz. At 4 kHz, the 725 mg E group had significantly less PTS than the 100 mg E group ($p = 0.05$).



In order to provide a perspective on the magnitude of protection afforded by systemic treatment with estradiol, the current results are compared to those from Hu et al. (1997), in which R-PIA was applied to the round window prior to noise exposure (see Poster # 165). The 725 mg dose of estradiol produced savings equivalent to those achieved with R-PIA. This is important because the protective effects achieved with R-PIA involved invasive surgery, whereas the protective effects of estradiol were achieved with simple systemic treatment.

3. There were no differences in hair cell loss after 4 kHz OBN noise exposure.

Figure 11. Hair cell losses (mean + sd). There were no remarkable differences among groups in IHC loss (dotted lines) or OHC loss (solid lines).



SUMMARY AND DISCUSSION

- Estradiol levels were determined from serum samples of female and male chinchillas, and found to vary on a continuum comparable to humans.

- The protective effects of estradiol on NIHL were demonstrated in two separate experiments that utilized different noise exposure conditions. Estradiol reduced NIHL from impulse noise simulating M16 rifle fire and octave band noise centered at 4 kHz.

- Estradiol was shown to have protective effects similar to another known otoprotectant, R-PIA (Hu et al., 1997). Unlike R-PIA, however, the protective effects of estradiol were achieved with simple systemic treatment rather than invasive surgery.

- Estradiol may exert its effects on the stria vascularis, or by acting as an antioxidant or a modulator of cochlear neurotransmitter substances. Like R-PIA (see Poster #165), steroid hormones can act through many different routes, each of which could be important for modulating susceptibility to NIHL. Estradiol can potentiate the activity of GABA; it can affect neuronal activity via changes in cellular neurochemistry and morphology; it can act on cell membranes to alter permeability to neurotransmitters, precursors and receptor functioning; it can act directly as an antioxidant; and it can influence the bioactivity of other antioxidants and blood flow promoters such as nitric oxide (1-4; 6,7).

- Based on our findings, it is reasonable to hypothesize that individuals with high estrogen levels will be less susceptible to NIHL than individuals with low estrogen levels.

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APPENDIX II

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PII Redacted

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EDUCATION:

WESTERN ILLINOIS UNIVERSITY, Macomb, Illinois:
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NORTHERN ILLINOIS UNIVERSITY, DeKalb, Illinois:
M.A. Psychology (1991), Neuroscience and Behavior
Thesis: *Responses of inferior colliculus neurons in young adult C57BL/6J mice as a function of stimulus azimuth.*
Ph.D. Psychology (1993), Neuroscience and Behavior
Dissertation: *Hearing in a noisy environment: Effects of noise location and sensorineural hearing loss on the responses of inferior colliculus neurons in C57BL/6J mice.*

CURRENT POSITION:

Research Associate Professor, Dept. of Communicative Disorders and Sciences, University at Buffalo, Buffalo, NY.
Visiting Assistant Professor, Dept. of Speech Pathology and Audiology, Fredonia College, Fredonia, NY.

PROFESSIONAL ORGANIZATIONS:

American Auditory Society; Association for Research in Otolaryngology; New York Academy of Sciences; Phi Kappa Phi Honorary Society; Sigma Xi Scientific Research Society; Society for Neuroscience.

AWARDS AND GRANTS:

Northern Illinois University Graduate School: Dissertation Completion Award/Research Fellowship, 1992-1993, $8,000.

U.S. Army Medical Research and Materiel Command: *Acquired Resistance to Impulse Noise*; Co-Investigator, 5/15/96-3/31/97, $192,630.

U.S. Army Medical Research Acquisition Activity: *Sex Differences in Susceptibility and Resistance to Noise-Induced Hearing Loss in Chinchillas*, Principal Investigator, 9/23/96-9/22/00, $481,021.

National Organization for Hearing Research: *Influence of SOD Deficiencies on Age-Related and Noise-Induced Cochlear Pathology and Hearing Loss in Mice*, Principal Investigator, 1/23/98-1/22/99, $10,000.

NIH: *Aging Auditory System: Prebycusis and its Neural Basis*, Program Project Grant, University of Rochester, *Animal Neurophysiology*, Consultant, 4/1/1998-3/31/2003.

NIH/NIDCD: *Acquired Hearing Loss*. Program Project Grant, Center for Hearing and Deafness, 1/1/99-12/31/04, $5,768,957.
Efferent Influences on Susceptibility to Cochlear Damage. Project Director, $136,417/yr.
Antioxidant Involvement in NIHL. Co-Director, $141,800/yr.
Animal Core, Project Director, $87,909/yr.

NIH: *Noise Induced Inner Ear Gene Expression,* R.T. Taggart, P.I., Co-Investigator (submitted).

NIH: *Cochlear Mechanisms in Noise-Induced Hearing Loss*, T. Nicotera, P.I., Co-Investigator (submitted).

PRE-DOCTORAL PROFESSIONAL AND RESEARCH EXPERIENCE:

Graduate Research Assistant for Dr. G.D. Coover (Biopsychology) and Dr. T. McCanne (Clinical Psychology), NIU (Spring, 1988).

Graduate Teaching Assistant, Introductory Psychology, NIU (Spring, 1988-Spring, 1990).

Administrative Assistant of Dr. Carlton E. Lints, Coordinator of Introductory Psychology Program, NIU (Fall, 1989-Spring, 1990).

Research conducted with Sharon A. Sandridge, Ph.D., Dept. of Communicative Disorders, NIU (Fall, 1991-Summer, 1992).

Graduate Research Assistant in the laboratory of James F. Willott, Ph.D., NIU (Summer, 1989; Summer, 1990-Summer, 1992). Involved in research using inbred mice (CBA/J and C57BL/6J) to study anatomical and electrophysiological changes in the central auditory system as a function of aging and sensorineural hearing loss.

GRADUATE COURSES COMPLETED (4.0 GPA):

NEUROSCIENCE: Biopsychology (PSY503), Psychopharmacology (PSY481), Neuroanatomy (PSY527, 528LAB), Neurophysiology (PSY529), Limbic System (PSY570E), Fundamentals of Sensory Perception (PSYC509), Neurobiology of Aging (PSY570E), Cell Neurophysiology (BIOS600A), Neurochemistry (PSY530).

RESEARCH TOOLS: Advanced Psychological Statistics (PSY504), Experimental Design (PSY506), Psychological Research (PSY590), Development of Educational Software for Microcomputers (LEIT439), Instrumentation and Software Development (PSY585).

LEARNING & DEVELOPMENT: Human Learning & Memory (PSY511), Adult Development and Aging (PSY425), Development of Language Acquisition (PSY575), Development of Perception and Learning (PSY577), Biopsychology of Learning (PSY570A).

MISCELLANEOUS PSYCHOLOGY: Clinical Analysis of Behavioral Pathology (PSY541), History of Psychology (PSY428), Practicum in College Teaching of Psychology (PSY581), Independent Research.

AUDIOLOGY: Hearing Problems and Audiometric Methods (COMD420), Anatomy & Pathology of the Ear (COMD527), Noise & Its Effects on Humans (COMD475), Psychosocial Aspects of Hearing Impairment (COMD528), Amplification Systems (COMD529), Audiological Assessment (COMD577), Electrophysiological Assessment of the Vestibular and Auditory System (COMD579T), Directed Research (COMD598).

POST-DOCTORAL PROFESSIONAL AND RESEARCH EXPERIENCE:

Postdoctoral Research Fellow (June, 1993-January, 1995), Developmental Auditory Physiology Laboratory, Boys Town National Research Hospital, Omaha, NE 68131.

Research Scientist (December, 1994-October, 1995), Hearing Research Laboratories, SUNY at Buffalo, Buffalo, NY.

Research Assistant Professor, Dept. of Communicative Disorders and Sciences (October, 1995-October, 1999), SUNY at Buffalo, Buffalo, NY.

Adjunct Clinical Instructor, Dept. of Communicative Disorders and Sciences (May, 1998-present), SUNY at Buffalo, Buffalo, NY.

Adjunct Research Assistant Professor, Dept. of Psychology (September, 1998-present), SUNY at Buffalo, Buffalo, NY.

Research Associate Professor, Dept. of Communicative Disorders and Sciences (October, 1999-present), SUNY at Buffalo, Buffalo, NY.

Visiting Assistant Professor, Dept. of Speech Pathology and Audiology (Fall, 2000), Fredonia College, Fredonia, NY.

TEACHING EXPERIENCE:

Student Teaching for Undergraduate Degree in Industrial Education, Bushnell Jr. High School, Bushnell, IL, 1978.

Industrial Arts Instructor, Litchfield Community School District, Litchfield, IL, 1979-1980.

Industrial Arts Instructor, Cook County School District, IL, 1980-1987.

Introductory Psychology, NIU, 1988-1990 (2 classes/semester).

Invited Lecturer, *Graduate Research Methods* (1995, 1998, 1999) and *Advanced Hearing Science* (1995, 1996, 1998, 1999), SUNY at Buffalo.
Developed and co-taught undergraduate course, *Anatomy and Physiology of Hearing*, SUNY at Buffalo, Spring, 1996, Spring 1999; Guest Lecturer, 1997.
Instructor, *Research Design in Speech Pathology and Audiology*, SH606, Fredonia College, Fredonia, NY, Fall 2000.

UNIVERSITY and PROFESSIONAL SERVICE:

Supervised thesis projects for S. Levine, M.A., SUNY at Buffalo, 1995-1996, and for N. Quaranta, M.D., Univ. of Bari, Italy, 1995-1996.
Supervised research project for Y. Min, M.D. (Otolaryngology resident), 1996.
Thesis Committee Member, Phillip J. Hofstetter, M.A.: *Effects of carboplatin on distortion product otoacoustic emissions and inner and outer hair cells of the chinchilla*, Spring, 1996.
Thesis Committee Member, Carrie A. Secor, M.A.: *Toneburst and derived-band responses from the inferior colliculus of noise exposed chinchillas*, Spring, 1998.
Statistical Consultant, Dr. Kim Tillery, 1997; Dr. Bridget Russell, 1997; Dr. Karen Yenser, 1997.
Sponsor, *Independent Study*, SUNY at Buffalo, Spring, 1997; Summer, 1997; Summer, 1999; Fall, 1999.
Dissertation Committee Member and project supervisor, Nancy Hight: *Noise induced hearing loss protection from GEE and R-PIA* (in progress).
Dissertation Committee Member, Jian Wang, PhD: *Modulation of neuronal behavior by GABA antagonists in the auditory cortex of chinchillas.*
Reviewer, *Audiology and Neuro-Otology, Brain Research, Hearing Research, Journal of Speech, Language, and Hearing Research, Gene Therapy,* and external reviewer for NIOSH.

BOOK CHAPTERS and REVIEW ARTICLES *(in chronological order):*

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R.F. Burkard (eds.), Annals of the New York Academy of Sciences, Vol. 884, Ototoxicity: Basic Science and Clinical Applications. NY: New York Academy of Sciences, pp. 152-170.

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10. **McFadden**, S.L. (2000) Genetics and age-related hearing loss. In: P.R. Hoff, and C.V. Mobbs (eds.), Functional Neurobiology of Aging, Chapter 41. Academic Press, pp. 597-603 (in press).
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14. Ding, D.L., **McFadden**, S.L., and Salvi, R.J. Cochlear hair cell densities and inner ear staining techniques. In: J.F. Willott (Ed.), Handbook of Mouse Auditory Research: From Behavior to Molecular Biology (submitted).
15. **McFadden**, S.L, Ohlemiller, K.K., Ding, D.L., and Salvi, R.J. The role of superoxide dismutase in age-related and noise-induced hearing loss: Clues from *Sod1* knockout mice. In: J.F. Willott (Ed.), Handbook of Mouse Auditory Research: From Behavior to Molecular Biology (submitted).

16. Willott, J.F., and **McFadden**, S.L. Age-related and noise-induced hearing loss. In L. Luxon and D. Prascher (Eds.), Biological Basis of Noise-Induced Hearing Loss (submitted).
17. **McFadden**, S.L., Ohlemiller, K.K., Ding, D.L., Shero, M., and Salvi, R.J. Influences of superoxide dismutase and glutathione peroxidase deficiencies on noise-induced hearing loss in mice. Proceedings of NOPHER 2000: An International Symposium on Noise Induced Hearing Loss, Cambridge, UK. (submitted).
18. Taggart, R.T., **McFadden**, S.L., Ding, D.L., Henderson, D., Sun, W., and Salvi, R.J. Gene Expression Changes in Chinchilla Inner Ear from Noise-Induced Temporary Threshold Shift. Proceedings of NOPHER 2000: An International Symposium on Noise Induced Hearing Loss, Cambridge, UK. (submitted).

PUBLICATIONS in PEER-REVIEWED JOURNALS (*in chronological order*):

1. Willott, J.F., Bross, L.S., and **McFadden**, S.L. (1992) Morphology of the dorsal cochlear nucleus in C57BL/6J and CBA/J mice across the life span. *Journal of Comparative Neurology* 321, 666-678.
2. Willott, J.F., Aitkin, L.M., and **McFadden**, S.L. (1993) Plasticity of primary auditory cortex (AI) associated with sensorineural hearing loss in adult C57BL/6J mice. *Journal of Comparative Neurology* 329, 402-411.
3. **McFadden**, S.L., and Willott, J.F. (1994a) Responses of inferior colliculus neurons in C57BL/6J mice with and without sensorineural hearing loss: Effects of changing the azimuthal location of an unmasked pure-tone stimulus. *Hearing Research* 78, 115-131.
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5. Willott, J.F., Bross, L.S., and **McFadden**, S.L. (1994a) Morphology of the cochlear nucleus in CBA/J mice with chronic, severe sensorineural cochlear pathology induced during adulthood. *Hearing Research* 74, 1-21.
6. Willott, J.F., Bross, L.S., and **McFadden**, S.L. (1994b) Morphology of the inferior colliculus in C57BL/6J and CBA/J mice across the life span. *Neurobiology of Aging* 15, 175-184.
7. **McFadden**, S.L., Walsh, E.J., and McGee, J. (1996) Onset and development of auditory brainstem response thresholds in the Mongolian gerbil (*Meriones unguiculatus*). *Hearing Research* 100, 68-79.
8. Hu, B.H., Zheng, X.Y., **McFadden**, S.L., Kopke, R., and Henderson, D. (1997) R-PIA attenuates noise-induced hearing loss in the chinchilla. *Hearing Research* 113, 198-206.
9. **McFadden**, S.L., Campo, P., Quaranta, N., and Henderson, D. (1997) Age-related decline of auditory function in the chinchilla (*Chinchilla laniger*). *Hearing Research* 111, 114-126.

10. **McFadden**, S.L., Henderson, D., and Quaranta, A. (1997) Remote masking in normal-hearing and noise-exposed chinchillas. *Audiology and Neuro-Otology* 2, 128-138.
11. **McFadden**, S.L., Henderson, D., and Shen, Y.H. (1997) Low-frequency 'conditioning' exposures provide long-term protection from noise-induced hearing loss in chinchillas. *Hearing Research* 103, 142-150.
12. **McFadden**, S.L., Quaranta, N., and Henderson, D. (1997) Suprathreshold measures of auditory function in the aging chinchilla. *Hearing Research* 111, 127-136.
13. Quaranta, A., **McFadden**, S.L., Henderson, D., and Sallustio, V. (1997) Remote masking in noise-exposed chinchillas. *Acta Oto-Laryngologica* (Stockh) 117, 226-228.
14. Zheng, X.Y., Ding, D.L., **McFadden**, S.L. and Henderson, D. (1997) Evidence that the inner hair cells are the major source of cochlear summating potentials. *Hearing Research* 113, 76-88.
15. Zheng, X.Y., Henderson, D., Hu, B.H., Ding, D.L., and **McFadden**, S.L. (1997) The influence of the cochlear efferent system on chronic acoustic trauma. *Hearing Research* 107, 147-159.
16. Zheng, X.Y., Henderson, D., Hu, B.H., and **McFadden**, S.L. (1997) Recovery of structure and function of inner ear afferent synapses following kainic acid excitotoxicity. *Hearing Research* 105, 65-76.
17. Zheng, X.Y., Henderson, D., **McFadden**, S.L, and Hu, B.H. (1997) The role of the cochlear efferent system in acquired resistance to noise-induced hearing loss. *Hearing Research* 104, 191-203.
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